SOME FACTORS WHICH AFFECT THE GROWTH OF *LACTOBACILLUS CASEI*

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SUMMARY.

A study has been made of the growth response of *Lactobacillus casei* to graded amounts of folic acid, riboflavin and Ca pantothenate in an assay medium designed for the measurement of folic acid and in modifications of that medium.

Growth was measured as titratable acidity after 72 hours' incubation and as turbidity after 18, 24 or 48 hours' incubation. The following conclusions were drawn from titration experiments.

Asparagine (720 mg./litre) increased the growth response to folic acid but reduced the growth response to riboflavin. The response to the lower concentrations of Ca pantothenate was reduced and to the higher concentrations was increased by asparagine.

In the folic acid assay (+ asparagine) and the riboflavin assay (− asparagine) the effect of reducing the concentration of purines and pyrimidines was to reduce the growth response. In the pantothenate assay, on the other hand, the response to the lower standard levels of Ca pantothenate was markedly increased by reducing the purine and pyrimidine concentration.

In the folic acid assay increasing the folic acid concentration from 0·4 mμg./ml. to 20 mμg./ml. had no effect on growth. In the riboflavin assay, however, growth was less with 20 mμg./ml. folic acid than with 0·4 mμg./ml. whether asparagine was present or not. In the pantothenate assay growth in the medium containing asparagine was less with 20 than with 0·4 mμg./ml. of folic acid (or equivalent concentrations of Ca leucovorin).

The response of *L. casei* to coenzyme A treated with alkaline phosphatase and pigeon liver enzyme or with phosphatase alone was compared with the response to Ca pantothenate. In the medium containing the highest concentration of purines and pyrimidines all the growth curves showed the characteristic S-shape. On reducing the concentration of purines and pyrimidines to one-eighth there was a marked increase in the response to all the lower dose levels.

INTRODUCTION.

The micro-organism *Lactobacillus casei* has been used for some years in this laboratory for the measurement of folic acid (Dawbarn, Hine and Smith, 1958). In order to adapt the procedure for the measurement of riboflavin and pantothenic acid either riboflavin or calcium pantothenate was omitted from the assay medium and folic acid was added to it. The response of the organism to graded amounts of the three substances was measured as titratable acidity

produced after 72 hours' incubation and was expressed in the usual way as dose response curves. It was found that the three curves differed markedly from one another in shape and therefore further study of the subject was undertaken.

EXPERIMENTAL.

The organism was obtained from the American Type Culture Collection in 1954 and has been maintained both in lyophilised cultures and by weekly subculture in the following medium.

Skim milk powder	... 50 gm.
Difco-bacto yeast extract	... 2·5 gm.
Evans hepamino	... 1·25 gm.
Vitamin B_{12}	... 5 μg.

Water to 500 ml., adjusted to pH 6·8 and autoclaved for 15 min. at 15 lb. pressure.

This medium is also used for cultures of *L. leichmannii*.

The assay medium which was used as a starting point in the investigation was a modification of that recommended by Toepfer, Zook, Orr and Richardson (1951) for the measurement of folic acid. Its composition is given in Table 1.

TABLE 1.

Composition of assay media

Folic acid assay medium.

Medium 1 for riboflavin and pantothenate assays.

Constituent	in 1 litre of medium
HCl hydrolysed casein	14·4 gm.
Glucose	48·0 gm.
Na acetate $\cdot 3H_2O$	77·0 gm.
DL-Tryptophane	240·0 mg.
L-Cystine	240·0 mg.
L-Asparagine	720·0 mg.
Adenine sulphate	12·0 mg.
Guanine HCl	12·0 mg.
Xanthine	12·0 mg.
Uracil	12·0 mg.
p-Aminobenzoic acid	1·0 mg.
Pyridoxine HCl	4·0 mg.
Pyridoxal HCl	4·0 mg.
Thiamine	0·4 mg.
Nicotinic acid	0·8 mg.
Biotin	20·0 μg.
Folic acid[1]	20·0 μg.
Riboflavin[2]	1·0 mg.
Ca pantothenate[3]	0·8 mg.
Thioglycolic acid	80·0 mg.
KH_2PO_4	1·78 g.
$MgSO_4 . 7H_2O$	400·0 mg.
$FeSO_4 . 7H_2O$	20·0 mg.
$MnSO_4 . 4H_2O$	264·0 mg.
Tween 80, 10 p.c.	2·0 ml.

[1] omitted in the folic acid assay
[2] omitted in the riboflavin assay
[3] omitted in the pantothenate assay

The inoculum medium consisted of the assay medium at half strength and containing 0·1 p.c. of a commercial liver preparation "hepamino" Evans. The folic acid, riboflavin and pantothenate content of the inoculum media are given in Table 2.

TABLE 2.

Folic acid, riboflavin and Ca pantothenate in inoculum media.

μg. per 100 ml. final medium.

Constituent	Folic acid assay	Riboflavin assay	Pantothenate assay
Folic acid:			
from hepamino*	0·46	0·46	0·46
from assay medium	—	1·00	1·00
total	0·46	1·46	1·46
Riboflavin:			
from hepamino*	17	17	17
from assay medium	50	—	50
total	67	17	67
Ca pantothenate:			
from hepamino*	21	21	21
from assay medium	40	40	—
total	61	61	21

* Amount available to the micro-organism; no attempt was made to liberate bound forms of the vitamin.

The organism was incubated for 18 hours in the inoculum medium and was then centrifuged, washed twice with saline and resuspended in saline to give a standard opacity. One drop of the final suspension was used to inoculate each assay tube.

In the work to be described the three types of assay were at times carried out simultaneously and the same inoculum was used for all. This was grown in the folic acid inoculum medium and for the sake of uniformity this inoculum medium was also used for most of the first part of the investigation. The possibility that the composition of the inoculum medium might affect the growth response of the organism in the assay was considered. However, the choice of inoculum medium had little effect on the shape of the dose response curves (titration procedure).

The following inoculum media were compared: in the pantothenate assay, media 1 and 3, Table 2; in the riboflavin assay, media 1 and 2, Table 2, and also an inoculum medium prepared from a riboflavin assay medium containing no asparagine and with 0·4 mμg./ml. of folic acid instead of 20 mμg./ml. In part 6 inoculum media containing high and low purine and pyrimidine concentrations were compared.

The assays were carried out in pyrex test tubes 17 mm. in internal diameter and 150 mm. in length. Each contained 5 ml. of assay medium and 5 ml. of a solution of folic acid, riboflavin or Ca-D-pantothenate. Four replicates of each concentration level of these standard solutions were employed. The tubes were plugged with cottonwool and autoclaved and were later inoculated and incubated at 37° C. Growth response to graded levels of the three substances was assessed either by measuring titratable acidity after 72 hours' incuba-

tion or by measuring turbidity after 18, 24 or 48 hours' incubation. In the former case autoclaving was for 10 minutes at 15 lb. pressure, in the latter the autoclave was turned off on reaching pressure. This was done to prevent darkening of the solutions. During autoclaving and incubation the tubes were arranged in random order, each set of replicates being randomized separately.

After some preliminary trials certain constituents of the media were selected for further study.

1. The effect of asparagine on the growth response of the organism to folic acid, riboflavin or Ca pantothenate was determined by a comparison of media containing no asparagine or 720 mg. asparagine per litre.

2. The effect of reducing the concentration of folic acid was examined in the riboflavin and pantothenate assays and, in the pantothenate assay only, the effect of substituting Ca leucovorin for folic acid.

3. In the three types of assay a comparison was made of media with different concentrations of purines and pyrimidines. The original medium contained equal weights of adenine sulphate, guanine HCl, xanthine and uracil. These were replaced by equimolecular amounts of purines and pyrimidines. Xanthine was omitted altogether. Solutions were prepared containing M/1000 adenine, guanine, uracil and thymine (No. 1) or M/1000 adenine and guanine and M/500 uracil (No. 2). These solutions were included in media in the following amounts: 48 ml., 24 ml., 12 ml., and 6 ml. per litre.

The concentration of purines and pyrimidines in the original medium and in media containing 48 ml./litre of solutions 1 or 2 was as follows:

Constituent	Amount in 1 litre of assay medium mg.		
	Original	with 48 ml. of solution 1	with 48 ml. of solution 2
Adenine sulphate	12	11·1	11·1
Guanine HCl	12	9·0	9·0
Uracil	12	5·4	10·8
Thymine	—	6·0	—
Xanthine	12	—	—

4 and 5. The effects of increasing the concentration of casein hydrolysate and of phosphate were considered in the folic acid and pantothenate assays.

6. The growth response of *L. casei* to the split products of coenzyme A was measured in pantothenate assay media containing low and high concentrations of purines and pyrimidines. The solution of CoA (N.B.C.) was split by alkaline phosphatase only or by alkaline phosphatase and a pigeon liver enzyme preparation (Novelli and Schmetz, 1951). The alkaline phosphatase (Sigma, purified) was dialyzed overnight against cold 0·02M ammonium acetate. A suspension of pigeon liver acetone powder (Sigma) was shaken up once with a preparation of Dowex 1-X8 (Novelli and Schmetz, 1951; Novelli, 1955).

The CoA solution treated with both enzyme preparations should contain free pantothenate and the other split products of CoA. When only alkaline phosphatase was used pantetheine and not pantothenate should be liberated.

RESULTS.

Three typical growth response curves obtained by the titration method and using the original media (Table 1) are shown in Fig. 1. The flatness of the riboflavin curve compared with that for folic acid and the "S-shape" of the curve for Ca pantothenate are obvious. Turbidity measurements in the folic acid assay are graphically presented in Fig. 2.

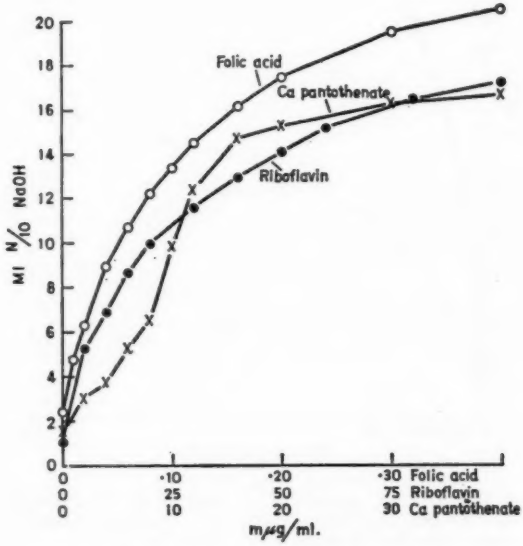

Fig. 1. The response of *L. casei* to graded amounts of folic acid, riboflavin or Ca pantothenate (medium in Table 1). Titratable acidity after 72 hours' incubation.

The effect of asparagine and of folic acid concentration.

Folic acid assay.

It is clear from Fig. 3 that the response of *L. casei* to graded amounts of folic acid was enhanced at every concentration level except the highest by the presence of asparagine.

When the concentration of folic acid was increased beyond the highest level used in the assay, i.e. 0·4 mμg./ml. there was no change in the response. At concentrations of 0·4, 0·8, 2·0, 8·0, 20·0 mμg./ml. the acidity produced per tube was ≡ 21 ml. N/10 NaOH and at these concentrations asparagine was without any effect (Table 3).

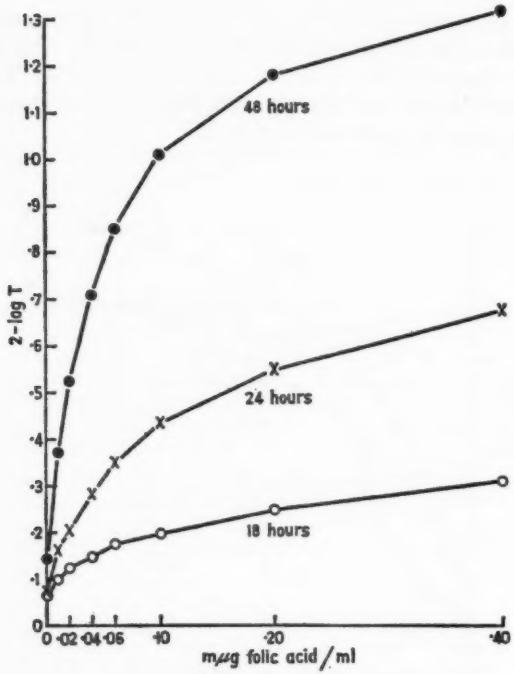

Fig. 2. The response of *L. casei* to graded amounts of folic acid (medium in Table 1). Turbidity measurements after 18, 24 or 48 hours' incubation.

TABLE 3.

The effect of increasing concentrations of folic acid in the folic acid assay. Titratable acidity (ml. N/10 NaOH per tube)

Folic acid mμg./ml.	Medium with asparagine	Medium without asparagine
0·40*	20·7	20·9
0·80	21·3	21·4
2·0	21·2	21·2
8·0	21·4	21·1
20·0	21·4	21·0

*=top standard.

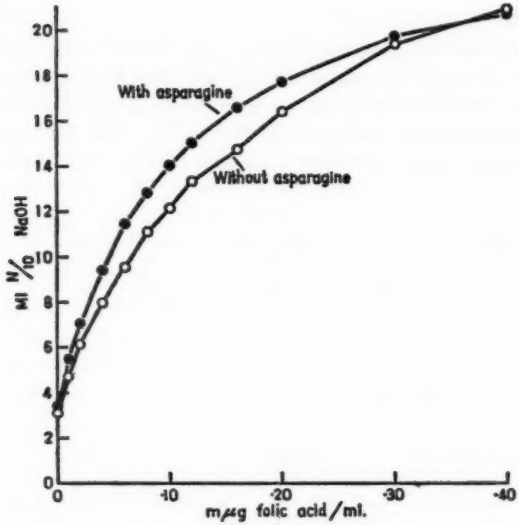

Fig. 3. The effect of asparagine on the response of *L. casei* to graded amounts of folic acid. Titratable acidity after 72 hours' incubation.

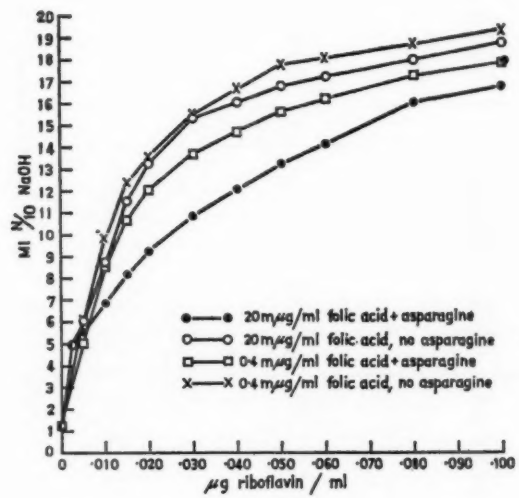
20 mμg/ml folic acid + asparagine
20 mμg/ml folic acid, no asparagine
0·4 mμg/ml folic acid + asparagine
0·4 mμg/ml folic acid, no asparagine
Ml N/10 NaOH
μg riboflavin / ml

Fig. 4. The effect of asparagine and of folic acid concentration on the response of *L. casei* to graded amounts of riboflavin. Titratable acidity after 72 hours' incubation.

Riboflavin assay.

Media with and without asparagine were tested with two concentrations of folic acid, i.e. 20 mμg./ml. and 0·4 mμg./ml. The higher concentration is that given in Table 1 and was adopted when the folic acid assay was modified for measurement of riboflavin and pantothenic acid. The lower concentration is 1/50th of this and is equal to the top standard in the folic acid assay.

TABLE 4.

The effect of asparagine and of folic acid concentration on the response of L. casei *to graded levels of riboflavin.*

Turbidity measurements (2—*Log T*).

Riboflavin μg./ml.	Media			
	0·4 mμg. folic acid/ml.		20 mμg. folic acid/ml.	
	No asparagine A	+ asparagine B	No asparagine C	+ asparagine D
	18 hours			
0	·02	·01	·01	·01
·0025	·15	·24	·15	·26
·005	·19	·30	·16	·41
·010	·21	·34	·18	·53
·020	·23	·35	·21	·60
·030	·22	·39	·21	·65
·050	·20	·36	·21	·65
·100	·21	·37	·21	·69
	24 hours			
0	·03	·02	·02	·02
·0025	·34	·28	·33	·20
·005	·48	·46	·49	·30
·010	·60	·58	·62	·37
·020	·70	·68	·72	·60
·030	·73	·74	·80	·70
·050	·74	·80	·86	·85
·100	·68	·87	·87	1·08
	48 hours			
0	·03	·02	·01	·02
·0025	·12	·10	·11	·05
·005	·23	·19	·22	·11
·010	·54	·46	·50	·20
·020	·73	·67	·74	·47
·030	·85	·83	·87	·61
·050	1·06	1·02	1·02	·74
·100	1·17	1·17	1·26	1·03

In the titrimetric assay the growth response was increased either by omitting asparagine from the medium or by reducing the concentration of folic acid from 20 mμg./ml. to 0·4 mμg./ml. When both these changes were made the growth curve was still higher (Fig. 4).

When growth response was measured by turbidity the maximum growth after 18 hours' incubation occurred in the original medium. However, this same medium gave much the lowest growth after 48 hours (Table 4 and Fig. 5).

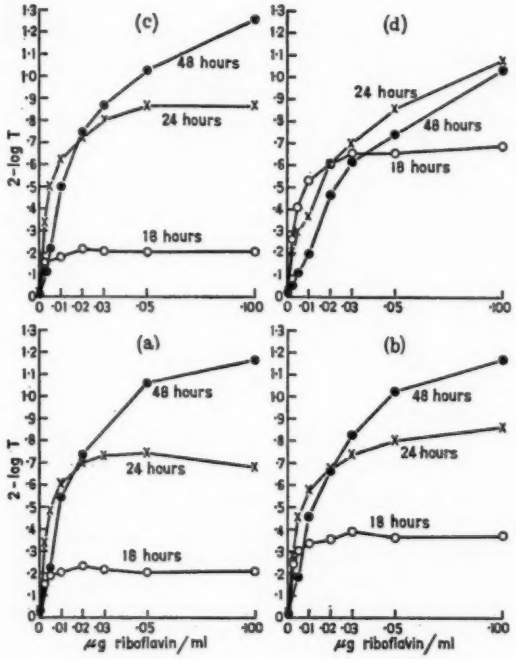

Fig. 5. The effect of asparagine and of folic acid concentration on the response of *L. casei* to graded amounts of riboflavin. Turbidity measurements after 18, 24 or 48 hours' incubation.

a No asparagine. 0·4 mμg. folic acid/ml.
b + asparagine. 0·4 mμg. folic acid/ml.
c No asparagine. 20 mμg. folic acid/ml.
d + asparagine. 20 mμg. folic acid/ml.

Pantothenate assay.

In this assay also a study was made of the effect of omitting asparagine from the medium and of reducing the concentration of folic acid from 20 mμg./ml. to 0·4 mμg./ml. The effect of substituting an equivalent amount of Ca leuco-

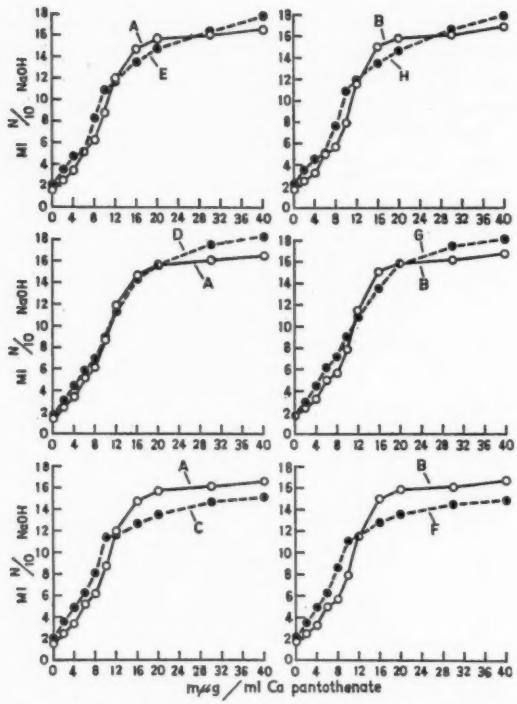

Fig. 6. The effect of asparagine and of folic acid or Ca leucovorin concentration on the response of *L. casei* to graded amounts of Ca pantothenate. Titratable acidity after 72 hours' incubation.

Left side: Folic acid in medium.		*Right side*: Ca leucovorin in medium.	
A	+ asparagine 20 mμg. folic acid/ml.	B	+ asparagine Ca leucovorin ≡ 20 mμg. folic acid/ml.
C	No asparagine 20 mμg. folic acid/ml.	F	No asparagine Ca leucovorin ≡ 20 mμg. folic acid/ml.
D	+ asparagine 0·4 mμg. folic acid/ml.	G	+ asparagine Ca leucovorin ≡ 0·4 mμg. folic acid/ml.
E	No asparagine 0·4 mμg. folic acid/ml.	H	No asparagine Ca leucovorin ≡ 0·4 mμg. folic acid/ml.

vorin for folic acid was included in the study. Ca leucovorin was earlier found to be equivalent to 38 p.c. of its weight of folic acid for the growth of *L. casei* in the folic acid assay (Dawbarn *et al.*, 1958).

The results of the tests are presented in Fig. 6. It is apparent that the substitution of Ca leucovorin for folic acid had no effect at either concentration whether asparagine was present or not.

Omitting asparagine from the medium containing 20 mμg./ml. of folic acid (or its equivalent of Ca leucovorin) resulted in increased growth with the lower concentrations of Ca pantothenate but reduced the response to the higher concentrations (Fig. 6: C and A, F and B).

Reducing the concentration of folic acid or Ca leucovorin, with asparagine in the medium, had the effect of increasing somewhat the growth response to both lower and higher levels of Ca pantothenate. The S-shape of the curve was less obvious than with the original medium (Fig. 6: D and A, G and B).

Three media were compared by the turbidimetric procedure; i.e., medium 1, containing asparagine and 20 mμg./ml. of folic acid; medium 2, containing 20 mμg./ml. of folic acid, but no asparagine; medium 3, containing asparagine and 0·4 mμg./ml. of folic acid.

After 18 hours the medium without asparagine (2) gave much the lowest growth. Medium 1 was better than medium 3 except at the lowest levels of Ca pantothenate. After 48 hours the relative position of the three curves resembled that in the titrimetric procedure.

The effect of the concentration of purines and pyrimidines.

Pantothenate assay.

The effect on the growth response to Ca pantothenate of including progressively lower amounts of adenine, guanine, uracil and thymine was determined by the titrimetric procedure in media containing 20 μg./litre of folic acid, with and without asparagine.

The results of these experiments are presented in Table 5 and, for the media containing asparagine, in Fig. 7.

In the media containing asparagine the reduction in the concentration of purines and pyrimidines caused a progressive increase in growth response to the lower standard levels. The optimal amount was 6 ml. per litre of medium for standard concentrations of Ca pantothenate up to 6 mμg./ml. However, as the concentration of Ca pantothenate rose above this level so did the optimal concentration of the purine-pyrimidine solution, first 12 then 24 and finally 48 ml./l. giving maximum growth response. It is of interest that when the medium was completely devoid of purines and pyrimidines the blank value rose to 4-5 ml. N/10 NaOH; the maximum response to Ca pantothenate was equivalent to nearly 15 ml. N/10 NaOH.

TABLE 5.

The effect of purine and pyrimidine concentration on the response of L. casei *to graded levels of Ca pantothenate.*

(Titratable acidity after 72 hours' incubation ml. N/10 NaOH per tube.)

Ca pantothenate mμg./ml.	0*	6*	12*	24*	48*
Part 1 +asparagine					
0	4·4	2·8	2·1	1·8	1·8
2	4·8	4·9	3·6	3·0	2·7
4	6·1	7·0	5·3	3·6	3·3
6	7·3	9·6	7·8	6·4	4·9
8	8·2	11·3	11·7	8·9	7·4
10	—	12·9	13·1	13·1	11·0
12	10·9	13·5	13·9	14·2	13·3
16	—	14·3	14·9	14·9	14·9
20	13·9	14·6	14·8	15·5	15·3
40	14·6	16·0	16·2	16·3	16·8
Part 2 −asparagine					
0	4·6	3·1	2·7	2·5	2·5
2	5·4	6·2	4·4	3·8	3·6
4	6·0	8·1	7·5	5·3	5·0
6	6·6	9·9	9·5	8·6	7·2
8	6·9	10·5	11·8	11·3	10·2
10	—	11·2	12·1	12·4	12·2
12	7·9	11·7	12·6	13·0	13·1
16	—	12·1	13·1	13·7	13·5
20	9·3	12·7	13·6	14·0	13·8
40	11·2	13·9	14·8	14·9	14·7

* ml. M/1000 adenine, guanine, uracil, thymine per litre of medium.

In the media without asparagine the results were somewhat similar. Growth at the lower standard levels was even greater than that obtained in the presence of asparagine. The reverse was true at concentrations of Ca pantothenate greater than 8-10 mμg./ml.

Riboflavin assay.

Media containing 20 μg. of folic acid per litre with and without asparagine were employed. Varying volumes of M/1000 adenine, guanine, uracil and thymine solutions were included, i.e. 48, 24, 12, 6 or 0 ml. per litre. The results obtained for the medium without asparagine are presented graphically in Fig. 8. It is apparent that there was considerable growth in the medium which contained no purines or pyrimidines. However, growth was enhanced when they were included. The growth curve rose more and more steeply as the amount of the purine and pyrimidine solution was increased from 6 ml. per litre to 12, 24 and 48 ml. per litre.

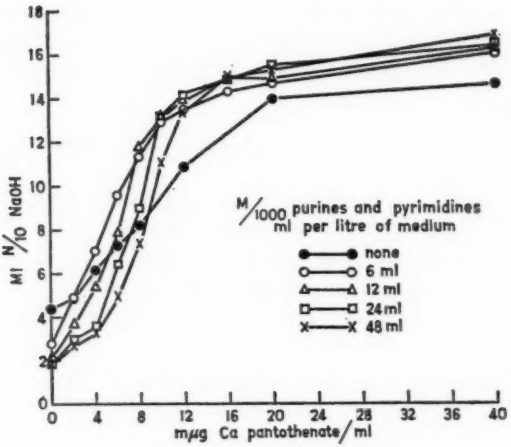

Fig. 7. The effect of the concentration of purines and pyrimidines on the response of *L. casei* to graded amounts of Ca pantothenate. Medium contained asparagine and 20 mμg. folic acid per ml. Titratable acidity after 72 hours' incubation.

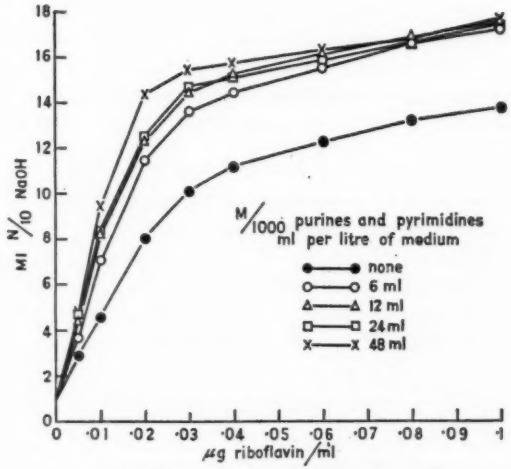

Fig. 8. The effect of the concentration of purines and pyrimidines on the response of *L. casei* to graded amounts of riboflavin. Medium contained 20 mμg. folic acid per ml. and no asparagine. Titratable acidity after 72 hours' incubation.

When the medium contained asparagine growth at most of the standard levels was greater with 12 or 24 ml. of the purine-pyrimidine solution per litre than with 6 ml. or 48 ml. The medium of choice was undoubtedly that containing 48 ml. of M/1000 adenine, guanine, uracil and thymine with no asparagine.

Folic acid assay.

Three mixtures of purines and pyrimidines were tested at four concentrations, i.e.:

No. 1: adenine, guanine, xanthine and uracil as in Table 1, and 50 p.c., 25 p.c. and 12·5 p.c. of these amounts.

No. 2: M/1000 adenine, guanine, uracil and thymine; 48 ml., 24 ml., 12 ml., and 6 ml. per litre of medium.

No. 3: M/1000 adenine and guanine, M/500 uracil; 48 ml., 24 ml., 12 ml. and 6 ml. per litre of medium.

In every case there was a progressive lowering of the growth response line as the amount of purines and pyrimidines was reduced. When these were omitted from the medium the growth response to the highest level of folic acid was ≡ 15 ml. N/10 NaOH.

The presence of thymine in the medium resulted in an increase in the blank (No. 2 above). When 48 ml. of the purine, pyrimidine solution were included in 1 litre of medium there was a blank ≡ 6-8 ml. N/10 NaOH. It is well known that partial growth of *L. casei* occurs in folic acid-deficient media which contain thymine and a purine (Stokstad, 1941; Krueger and Peterson, 1945; Prusoff, Teply and King, 1948; Hitchings, Elion, Falco, Russell, Sherwood and Vanderwerff, 1950). The methylation of uracil (or desoxyuridine 5′-phosphate) to thymine (or thymidine 5′-phosphate) is one of the functions of the folic acid coenzyme (Greenberg and Humphreys, 1958; Humphreys and Greenberg, 1958; Huennekens and Osborn, 1959; McDougall and Blakley, 1960).

The effect of nitrogen and phosphate.

The growth response to every level of Ca pantothenate could be enhanced beyond that attained on the medium containing the lowest concentration of purines and pyrimidines. This was done by increasing the concentration of casein hydrolysate by 50 p.c. or by including 5 times the usual amount of phosphate (as in the medium of Clegg, Kodicek and Mistry, 1952). When casein and phosphate were both increased still greater growth occurred.

The response of *L. casei* to graded amounts of folic acid also could be increased by raising the concentration of casein hydrolysate or phosphate in the medium.

Coenzyme A split by enzymes.

The response of *L. casei* to graded amounts of the split products of coenzyme A was determined in two media, one containing 48 ml., the other 6 ml. per

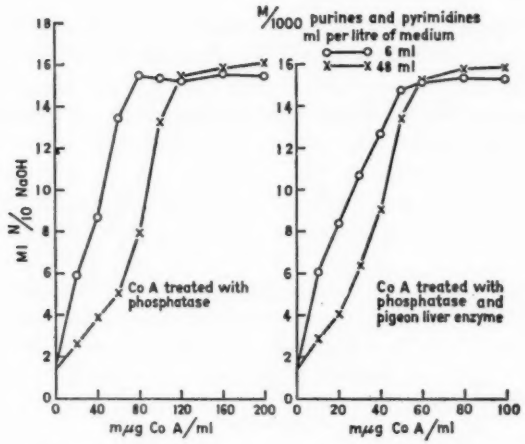
M/1000 purines and pyrimidines
ml per litre of medium
6 ml
48 ml
Ml N/10 NaOH
Co A treated with phosphatase
Co A treated with phosphatase and pigeon liver enzyme
mμg Co A/ml
mμg Co A/ml

Fig. 9. The effect of the concentration of purines and pyrimidines on the response of *L. casei* to graded amounts of the split products of coenzyme A. Medium contained asparagine and 20 mμg. folic acid per ml. Titratable acidity after 72 hours' incubation.

litre of M/1000 adenine, guanine, uracil and thymine. The results are presented graphically in Fig. 9. The S-shape of the curve for pantothenate on the medium with the higher concentration of purines and pyrimidines was repeated in the response curves for CoA split by alkaline phosphatase and pigeon liver enzyme and for CoA partially split by phosphatase alone. As with pantothenate so also with partially or completely split CoA, the reduction in the concentration of purines and pyrimidines led to a marked increase in the growth response at all lower dose levels.

DISCUSSION.

Lactobacillus casei has been grown in this laboratory in a medium designed to supply all its nutritional requirements. The omission of one essential nutrient and its separate provision in graded amounts formed the basis of an assay for that nutrient. This has been the standard procedure for carrying out microbiological assays for more than twenty years (Snell and Strong, 1939).

The medium we used was devised for the assay of folic acid but it was expected that it would be equally satisfactory for the assay of riboflavin or pantothenic acid. When this proved not to be the case a study was made of the effect of certain changes in the medium on the growth response of the organism to graded amounts of folic acid, riboflavin or Ca pantothenate.

Asparagine : folic acid concentration.

When folic acid was the growth-limiting factor, growth response to this substance, measured as titratable acidity after 72 hours' incubation, was increased by asparagine at every level except the highest. Extra folic acid up to 50 times the top standard concentration did not cause an increase or decrease of growth and with these high concentrations of folic acid asparagine was without effect.

When riboflavin was the growth-limiting factor asparagine had an inhibitory effect. So also had folic acid, growth being less with 20 mμg./ml. than with 0·4 mμg./ml.

When pantothenate was the limiting factor and the medium contained 20 mμg./ml. of folic acid asparagine was inhibitory at the lower levels of Ca pantothenate but stimulated growth at the higher levels. In the presence of asparagine folic acid was slightly inhibitory. The reduced formylated compound, Ca leucovorin, had the same effect in this assay as an equivalent amount of folic acid.

When growth was measured by turbidity the relative position of growth response curves was dependent on the time of incubation. In the riboflavin assay growth up to 18 hours was markedly stimulated by asparagine but when it was measured at 48 hours asparagine was inhibitory. Both stimulation and inhibition were increased in the presence of 20 mμg./ml. of folic acid as compared with 0·4 mμg./ml.

Asparagine also stimulated growth up to 18 hours in the pantothenate assay.

The scientific literature contains numerous reports of the effect of asparagine on the growth of *L. casei*. Kodicek and Mistry (1953) reported that asparagine inhibited the growth of *L. casei* measured after 72 hours' incubation but that it was somewhat stimulatory after 17 hours. When partially hydrolysed insulin ("strepogenin") was added to the medium as well as asparagine stimulation up to 17 hours was marked and inhibition at 72 hours was greatly reduced. Stokes, Koditschek, Rickes and Wood (1949) found that in the medium employed by them asparagine inhibited the growth of *L. casei* measured after 17 and 24 hours. The addition of trypsinized casein overcame the inhibition. Serine overcame the inhibition due to asparagine in the experiments of Rickes, Koch and Wood (1949).

As early as 1942 Feeney and Strong found that if asparagine were included in the medium for pantothenate assay a yeast supplement might be omitted. In the riboflavin assay, however, there was a decreased bacterial response in the presence of asparagine.

Purines and pyrimidines.

When Ca pantothenate was the growth-limiting factor a marked increase in the growth response to the lower standard levels occurred when the concen-

tration of purines and pyrimidines was reduced, the optimal amount of these rising with the pantothenate concentration. The sigmoidal shape of the growth response curve was almost eliminated by reducing the purine and pyrimidine concentrations to one-eighth.

No such relationship was apparent in the folic acid and riboflavin assays. In the folic acid assay and in the riboflavin assay when the medium contained no asparagine the growth response curve rose more steeply as the amount of purines and pyrimidines was increased.

In all three types of assay there was considerable growth when purines and pyrimidines were omitted from the medium.

The sigmoidal shape of the dose response curve in the pantothenate assay when the medium contained the highest concentration of purines and pyrimidines was repeated when completely or partially split coenzyme A was substituted for pure Ca pantothenate. Thus the provision of the other split products of CoA as well as pantothenic acid did not alter the shape of the dose response curve. Nor did the substitution of pantotheine for pantothenic acid improve the shape of the line.

As a result of the foregoing tests the original assay medium (Table 1) was modified for the measurement of riboflavin and pantothenic acid in natural materials.

Asparagine was omitted from the riboflavin assay medium but was included at the original concentration in the medium for pantothenate assay.

The original concentration of folic acid, 20 μg./litre, was maintained in both assay media. This amount should preclude interference by folic acid present in extracts of natural materials.

Purines and pyrimidines as listed in Table 1 could be retained in the riboflavin assay medium or replaced by 48 ml. per litre of M/1000 adenine, guanine, uracil and thymine. Only 6 ml. of this solution were included in each litre of pantothenate assay medium.

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THE EFFECT OF HEPATOTOXINS ON E_{260} VALUES OF LIVER AND OF SERUM

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SUMMARY.

The extinction coefficients at 260 mμ (E_{260}) of acid extracts of liver homogenates and of serum were measured in rats killed serially after dosing with the hepatotoxic pyrrolizidine alkaloid, lasiocarpine, or with lethal or non-lethal doses of carbon tetrachloride.

Both lasiocarpine and CCl_4 at either dose rate produced a fall in the E_{260} value of liver within 1-5 hours of administration and a concomitant rise in the E_{260} value of serum. The change in E_{260} values was more rapid with CCl_4 than with lasiocarpine and the rise in serum E_{260} was greater for a given fall in liver E_{260} value with CCl_4 than with lasiocarpine. Although lasiocarpine caused a slower change, the fall in E_{260} values for liver was eventually greater with the alkaloid than with either dose of CCl_4.

The loss from cells of cytoplasmic material which absorbs light at 260 mμ within 1-3 hours of dosing with CCl_4, long before the effect of mitochondrial damage becomes evident, supports the hypothesis that cellular injury is first exerted by CCl_4 at the cell membrane rather than at the mitochondrial membrane.

The inverse relationship between E_{260} values of liver and of serum for lasiocarpine indicates that this hepatotoxin, like CCl_4, increases the permeability to oxidised pyridine nucleotides of the cell membrane as well as of the mitochondrial membrane. The effect on cell permeability evidently occurs very soon after administration of the alkaloid.

It is suggested that the determination of the E_{260} values of acid extracts of serum might provide an early, simple test for the diagnosis and evaluation of certain types of toxic liver damage.

INTRODUCTION.

In an earlier study Gallagher and Rees (1960) found that an inverse relationship existed between the extinction coefficients at 260 mμ (E_{260}) of acid extracts of liver homogenates and of serum 9 hours after the administration to rats of the hepatotoxins carbon tetrachloride and dimethyl nitrosamine (DMN). The fall in liver E_{260} levels following dosing with either CCl_4 or DMN was accompanied by a rise in serum E_{260} values. However, when rats were poisoned with thioacetamide, another hepatotoxin, there was no significant change in the E_{260} values of either liver homogenates or serum. This result was not surprising as Gallagher, Gupta, Judah and Rees (1956) had shown that thioacetamide poisoning led to inactivation of respiratory enzymes

by producing an inhibitory accumulation of Ca^{++} within liver mitochondria and not by causing the loss of pyridine nucleotides from the mitochondria as is the case with CCl_4 and DMN. The E_{260} values of acid extracts of tissue may be taken as a measure of the concentration of oxidised pyridine nucleotides as Siekevitz and Potter (1953, 1955) have shown that the greatest proportion of light absorption at this wavelength is due to oxidised pyridine nucleotides.

Hepatotoxic pyrrolizidine alkaloids have been shown to lead to the loss of pyridine nucleotides from liver mitochondria both *in vivo* (Christie, 1958) and *in vitro* (Gallagher, 1960a). The present study was undertaken to determine (a) the possibility of a relationship between liver and serum E_{260} values in rats poisoned with the pyrrolizidine alkaloid, lasiocarpine, (b) the chronological development of liver-serum E_{260} relationships in CCl_4 and lasiocarpine poisoning and (c) the possible clinical use of serum E_{260} levels as an early measure of liver damage.

Materials and Methods.

Animals.

Male Wistar rats weighing 150-180 gm. and fed a normal stock diet were used throughout the experiments.

Reagents.

Carbon tetrachloride of reagent grade was further purified by distillation. Lasiocarpine was kindly supplied by Dr. C. C. J. Culvenor of the Chemical Research Laboratories, C.S.I.R.O.

Methods.

In order to measure the E_{260} values of serum and of liver rats were anaesthetised with diethyl ether and exsanguinated into centrifuge tubes from the severed carotid arteries. The livers were then removed rapidly and immersed in ice-cold 0·25 M sucrose solution. Homogenates of 2 gm. of liver in ice-cold 0·25 M sucrose were prepared immediately in glass homogenisers. Duplicate 0·5 ml. samples of 5 p.c. w/v homogenates were transferred quickly to glass centrifuge tubes containing 4·5 ml. of 5·56 p.c. trichloroacetic acid (TCA) at 0° C. to give a final volume of 5 ml. of 5 p.c. TCA. The tubes of blood were stood at 25° C. for 10 minutes, the clots freed with a glass rod and then stood at 0° C. for 30 minutes. Serum was taken off with Pasteur pipettes and duplicate 0·5 ml. samples were added to glass centrifuge tubes containing 4·5 ml. of 5·56 p.c. TCA. The tube contents of 5 p.c. TCA containing serum or liver were mixed with glass rods, allowed to stand at room temperature (20-24° C.) for 10 minutes and were then centrifuged at 2000 r.p.m. in an M.S.E. Multiplex centrifuge for 5 minutes. Each supernatant was collected and the E_{260} value was measured against a blank of 5 p.c. TCA in a Beckman model D.U. spectrophotometer. Dry weights of homogenates were determined by extracting 30 ml. samples three times with a mixture of boiling ethanol and diethyl ether (3 to 1, v/v) to remove lipids, drying in an oven at 100° C. for 24 hours and then cooling in a desiccator before weighing.

Undiluted CCl_4 was administered by a syringe attached to a polythene tube into the stomachs of rats anaesthetised with ether. The dose used was 3·5 ml./kg. or 1·1 ml./kg.

as indicated. Earlier studies (Gallagher, 1960b) had shown that the larger dose rate was almost always fatal. The smaller dose rate was chosen to determine the effect of a non-lethal dose on liver and serum E_{260} levels.

Lasiocarpine was dissolved in water by neutralising with N HCl and administered by intraperitoneal injection at the LD_{50} rate of 77 mg./kg. (Bull, Dick and McKenzie, 1958).

RESULTS.

Effect of lasiocarpine on E_{260} values of acid extracts of liver homogenates and serum.

A group of 40 rats was injected intraperitoneally with lasiocarpine. Four rats were bled and killed immediately they received lasiocarpine. The rest were killed in groups of four at the times shown in Table 1. Two rats only survived to 69 hours to form the last group.

TABLE 1.

Effect of an LD_{50} of lasiocarpine on E_{260} values of acid extracts of liver homogenates and serum.

Procedure: Trichloroacetic acid extracts of 0·5 ml. duplicate samples of 5 p.c. liver homogenates and of serum were prepared as described in the "Methods" section. 77 mg. lasiocarpine/kg. was administered to male rats by intraperitoneal injection of a neutral solution. Groups of 4 animals were killed at the times shown in the Table. Two animals only survived to 69 hours. The E_{260} readings have been corrected to 10 mg. of fat-free dry homogenate/sample, and to 1 ml. of serum/sample.

Time killed (hours after receiving lasiocarpine)	E_{260}			
	Liver		Serum	
	Range	Mean	Range	Mean
0	1·73-1·96	1·86	0·28-0·31	0·30
3	1·72-1·80	1·77	0·40-0·43	0·42
5	1·22-1·65	1·44	0·35-0·36	0·36
7	1·03-1·48	1·26	0·29-0·41	0·35
9	1·22-1·25	1·24	0·40-0·43	0·41
11	0·15-1·20	0·72	0·50-0·61	0·56
21	0·17-1·00	0·59	0·59-0·71	0·65
24	0·15-0·20	0·18	0·48-0·53	0·50
45	0·14-0·20	0·17	0·72-0·83	0·77
69	0·15-0·17	0·16	0·90-1·00	0·95

E_{260} values of liver homogenates.

Table 1 shows that there was a progressive decrease in the E_{260} values of acid extracts of liver homogenates following the administration of lasiocarpine. The fall was apparent at five hours after dosing, severe at 11 hours and very large at 24 hours and afterwards.

E_{260} values of serum.

As the absorption at 260 mμ is due to the pyridine ring and not to the entire pyridine nucleotide the fall in E_{260} values of extracts of homogenates of whole liver suggested that lasiocarpine caused a leakage of pyridine nucleotides from liver cells rather than the breakdown of the coenzymes within the cells. Measurement of the E_{260} values of extracts of serum confirmed this. The data presented in Table 1 show that there was a rapid rise in serum E_{260} which was apparent, although not large, three hours after giving lasiocarpine. The E_{260} value increased greatly from 11 hours onward and in the animals kept alive for 69 hours it reached three times that of the rats killed at injection time.

Relationship between liver and serum E_{260} values.

The inverse relationship between the E_{260} values of liver and serum extracts in lasiocarpine poisoning is shown in Fig. 1. As the liver E_{260} fell, the serum E_{260} rose, indicating that some at least of the pyridine nucleotides lost from liver as a result of lasiocarpine poisoning remain in the serum.

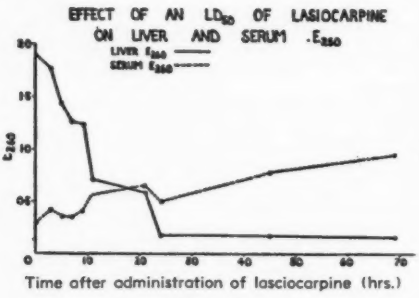

Fig. 1

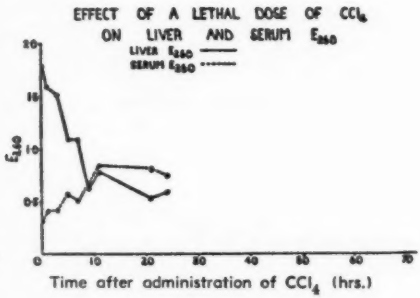

Fig. 2

Effect of a lethal dose of CCl_4 on E_{260} values of acid extracts of liver homogenates and serum.

A group of 40 rats was given a lethal dose of CCl_4 by stomach tube. Four rats were bled and killed immediately they received CCl_4. The rest were killed in groups of four at the times shown in Table 2. Another group of four rats died before 48 hours, the intended time of their slaughter.

E_{260} values of liver homogenates.

The administration of a lethal dose of CCl_4 produced a very rapid fall in the E_{260} values of acid extracts of liver (Table 2). The decrease was apparent an hour after giving CCl_4, considerable by five hours and very large from nine hours onward. The E_{260} values fell more quickly in rats which received CCl_4 than in those which received lasiocarpine (cf. Table 1) but did not drop to such low levels.

TABLE 2.

Effect of a lethal dose of CCl_4 on E_{260} values of acid extracts of liver and serum.

Procedure: As in Table 1 except that 3·5 ml. CCl_4/kg. was administered by stomach tube to male rats. Groups of 4 animals were killed at the times shown in the Table and the results are expressed as in Table 1.

Time killed (hours after receiving CCl_4)	E_{260}			
	Liver		Serum	
	Range	Mean	Range	Mean
0	1·65-1·88	1·80	0·29-0·32	0·31
1	1·54-1·68	1·60	0·38-0·44	0·41
3	1·33-1·72	1·52	0·40-0·43	0·42
5	1·12-1·24	1·19	0·56-0·59	0·57
7	1·17-1·20	1·19	0·45-0·56	0·51
9	0·62-0·65	0·63	0·57-0·79	0·68
11	0·62-0·93	0·78	0·75-0·93	0·84
21	0·48-0·58	0·53	0·81-0·84	0·82
24	0·53-0·68	0·60	0·76-0·86	0·78

E_{260} values of serum.

Table 2 also shows that there was a rapid change in serum E_{260} values as a result of CCl_4 administration. An appreciable rise occurred in an hour after the start of the experiment. By nine hours the E_{260} values had doubled. As with liver, the change in E_{260} values occurred more rapidly with CCl_4 than with lasiocarpine administration but did not quite reach the same magnitude.

Relationship between liver and serum E_{260} values.

A clear inverse relationship was apparent between the E_{260} values of liver and serum following the administration of CCl_4 as shown in Fig. 2.

Effect of a non-lethal dose of CCl_4 on E_{260} values of acid extracts of liver homogenates and serum.

In order to determine whether a small dose of CCl_4 produced changes of significant magnitude in E_{260} values 32 rats were given 1·1 ml. CCl_4/kg. by stomach tube. Four rats were bled and killed immediately after receiving CCl_4. The rest were killed in groups of four at the times indicated in Table 3.

E_{260} values of liver homogenates.

The smaller dose of CCl_4 produced a pattern of change in the E_{260} values of acid extracts of liver similar to that of the larger dose but less in degree (Table 3). There was no significant fall at an hour after dosing but from three hours onward the fall was considerable and at 24 hours the E_{260} value was less than 50 p.c. of pre-administration levels.

TABLE 3.

Effect of a non-lethal dose of CCl_4 on E_{260} values of acid extracts of liver homogenates and serum.

Procedure: As in Table 1 except that 1·1 ml. CCl_4/kg. was administered by stomach tube to male rats. Groups of 4 animals were killed at the times shown in the Table and the results are expressed as in Table 1.

Time killed (hours after receiving CCl_4)	E_{260}			
	Liver		Serum	
	Range	Mean	Range	Mean
0	1·61-1·92	1·76	0·28-0·35	0·32
1	1·51-1·84	1·68	0·29-0·37	0·33
3	1·28-1·45	1·37	0·63-0·66	0·64
5·5	1·25-1·31	1·28	0·67-0·77	0·72
24	0·60-0·98	0·80	0·46-0·81	0·63
26	0·60-0·63	0·62	0·64-0·80	0·72
28	0·68-0·97	0·82	0·56-0·77	0·67
48	0·53-0·96	0·76	0·81-0·92	0·87

E_{260} values of serum.

Table 3 shows that, as with liver at this dose of CCl_4, there was no change in E_{260} values an hour after administration but that a considerable change had occurred by three hours. From three hours onward the serum E_{260} values were double, or more, those of animals at the time of dosing.

Relationship between liver and serum E_{260} values.

It is shown in Fig. 3 that the inverse relationship between liver and serum values of E_{260} holds good also for the non-lethal dose of CCl_4.

Comparison of mean E_{260} values of liver homogenate and serum extracts in the three experimental groups.

Table 4 shows the corresponding mean serum E_{260} values for each of the experimental groups at points in a declining order of mean liver E_{260} values. It will be seen that in each group there was an inverse relationship between the concentration of materials absorbing light at 260 mμ in acid extracts of liver and serum. However, although there was good agreement between the serum E_{260} values for the same value of liver E_{260} with the two groups which received CCl_4, the serum E_{260} in the group injected with lasiocarpine was lower for a given level of liver E_{260} than in the groups poisoned with CCl_4.

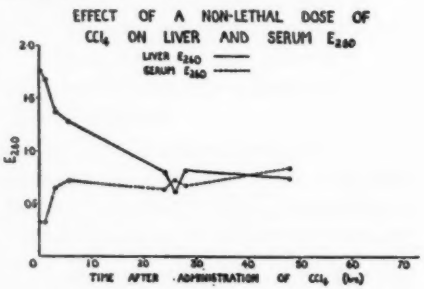

Fig. 3

TABLE 4.

Comparison of mean E_{260} values of acid extracts of liver homogenates and serum.

Liver E_{260}	Serum E_{260}		
	Lasiocarpine	CCl_4 (3·5 ml./kg.)	CCl_4 (1·1 ml./kg.)
1·80-1·90	0·30	0·31	—
1·70-1·80	0·42	—	0·32
1·60-1·70	—	0·41	0·33
1·50-1·60	—	0·42	—
1·40-1·50	0·36	—	—
1·30-1·40	—	—	0·64
1·20-1·30	0·35, 0·41	—	0·72
1·10-1·20	—	0·51, 0·57	—
0·80-0·90	—	—	0·63, 0·67
0·70-0·80	0·56	0·84	0·87
0·60-0·70	—	0·68, 0·78	0·72
0·50-0·60	0·65	0·82	—
0·10-0·20	0·50, 0·77, 0·95	—	—

DISCUSSION.

This study has shown that there is an inverse relationship between the E_{260} values of acid extracts of liver and of serum following the administration to rats of lethal or non-lethal doses of CCl_4 or of an LD_{50} of the hepatotoxic pyrrolizidine alkaloid, lasiocarpine. In each case there is a fall in the liver values and a concomitant rise in the values of serum. The results with respect to CCl_4 confirm an earlier observation of Gallagher and Rees (1960) who found evidence of a similar relationship between liver and serum E_{260} values in rats poisoned with another hepatotoxin, dimethyl nitrosamine; in this case also the E_{260} value of liver fell and that of serum rose, as with CCl_4 and lasiocarpine. In rats poisoned with thioacetamide, however, there was no change in either liver or serum E_{260} values despite the fact that the dose of thioacetamide administered would have killed many liver cells as a result of inhibition of mitochondrial respiration by the accumulation of Ca^{++} (Gallagher *et al.*, 1956).

The data presented show clearly that in both CCl_4 and lasiocarpine poisoning there is a considerable loss of oxidised pyridine nucleotides from liver cells and a corresponding rise of these materials in the serum long before the usual time of onset of liver necrosis. Consequently it cannot be argued that such changes in E_{260} values are merely secondary to the death of liver cells. The preservation of normal E_{260} values in rats poisoned with thioacetamide indicates that liver necrosis as such does not necessarily lead to a fall in the overall concentration of oxidised pyridine nucleotides in liver homogenates.

Rees and Sinha (1960) measured the activity in serum of certain enzymes subsequent to liver injury by CCl_4. They found that there was an increase in

isocitric dehydrogenase activity and in L-malic dehydrogenase activity in rat serum six hours after administering CCl_4 but no change in serum L-glutamic dehydrogenase until 12 hours after poisoning. Isocitric dehydrogenase is present largely in the supernatant fraction of liver cell preparations, and L-malic dehydrogenase is about equally distributed between the liver cell supernatant and mitochondria, whereas L-glutamic dehydrogenase is entirely mitochondrial. Consequently, following dosing with CCl_4, the much earlier appearance in the serum of the supernatant enzymes isocitric dehydrogenase and L-malic dehydrogenase than the exclusively mitochondrial L-glutamic dehydrogenase indicates that the initial injury in CCl_4 poisoning is at the cell membrane. This allows the escape of cytoplasmic constituents into the serum. Mitochondrial damage only occurs later. The present study supports this deduction as it reveals a loss of cytoplasmic acid-soluble material which absorbs light at 260 $m\mu$ 1-3 hours after administration of CCl_4, long before the effect of mitochondrial injury has become evident.

Loss of material-absorbing light at 260 $m\mu$ from homogenates of whole liver following poisoning with lasiocarpine and the accompanying rise in serum E_{260} values show that this hepatotoxin too, like CCl_4, produces an increase in the permeability of the cell membrane as well as of mitochondrial membranes. Gallagher (1960a) has presented evidence that the effect of lasiocarpine on mitochondrial permeability is associated with the charge carried on the ring nitrogen of the alkaloid. It seems likely that the cell membrane might similarly be affected by this charge with a resulting increased permeability to pyridine nucleotides.

It is suggested that the measurement of the E_{260} values of serum by the simple method outlined in this paper might provide an early ancillary diagnostic indication of the presence and of the degree of liver damage. The test is simpler than, and it should be at least as useful as, the determination of serum levels of transaminases provided that adequate data have been obtained to establish the normal range of serum E_{260} values for a given species.

Poisoning of rats with lethal or non-lethal doses of CCl_4 or with the pyrrolizidine alkaloid lasiocarpine produces a rapid rise in the E_{260} value of serum, apparent 1-3 hours after administering the drug. The rise in serum E_{260} value is inversely related to the E_{260} value of liver and it progresses as liver damage increases. There is good agreement between serum E_{260} values at a given value of E_{260} of liver in CCl_4 poisoning at either rate of dosing, but the serum E_{260} values tend to be lower in lasiocarpine poisoning.

Damage to cells in tissues other than the liver would quite likely lead to elevation of serum E_{260} values but this limitation applies also to the measurement of transaminase activity as a diagnostic test. Neither a rise in the serum concentration of materials absorbing light at 260 $m\mu$ nor of transaminases should be taken as pathognomonic of liver disease in the absence of supporting evi-

dence. The present technique may prove to be especially useful when applied to assess, at an early stage, the degree of liver damage in accidental or occupational poisonings with hepatotoxins.

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THE INTERACTION OF STAPHYLOCOCCAL TOXIN AND GANGLIOSIDE

I. INACTIVATION OF THE LETHAL EFFECT OF STAPHYLOCOCCAL TOXIN IN MICE

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SUMMARY.

Evidence is presented that staphylococcal toxin and serum proteins form firm combinations with ganglioside.

Ganglioside from which up to 62 p.c. of the sialic acid is removed by either heat or neuraminidase still strongly inactivates staphylococcal toxin.

Sedimentation data are presented for unheated and heated aqueous solutions of ganglioside. These data, together with inactivation studies, support the idea that heated aqueous solutions form smaller or unfolded aggregates with more active sites available for interaction with toxins.

Mechanisms of combination between gangliosides and proteins are discussed.

INTRODUCTION.

van Heyningen (1959) tentatively identified ganglioside as the tetanus toxin receptor in nervous tissue. More recently it was reported that staphylococcal, tetanus and diphtheria toxins and also the toxins of *Cl. welchii* type A (*perfringens*) and type D (*ovitoxicum*) were inactivated by ganglioside *in vitro* (North and Doery, 1961; North, Pawlyszyn and Doery, 1961).

It was found that the degree of inactivation depended on the time of contact between toxin and ganglioside and that it was increased if the ganglioside was previously heated in a water bath at 100° C. and, further, that ganglioside failed to inactivate toxin in the presence of albumin.

In this paper further studies concerning the nature of the interaction of bacterial toxins and ganglioside will be described.

MATERIALS AND METHODS.

Ganglioside.

Crude ganglioside was prepared from the grey matter of ox brain by a method essentially that described by Bogoch (1958). This was purified by distribution in the proportions of 100 mg. between $CHCl_3$ – methanol (2:1 v/v), 20 ml. and 0·1 M NaCl, 5 ml. The aqueous layer was subjected to prolonged dialysis against distilled water then freeze dried. Aqueous solutions of different preparations of ganglioside (1 mg./ml.) varied in pH between

4·3 and 5·9. The pH values above 4·3 are now attributed to trace amounts of ammonia introduced during dialysis. The preparation used in the experiments reported here contained 29 p.c. sialic acid (as N-acetyl neuraminic acid), 21 p.c. hexose (as galactose), 7·0 p.c. hexosamine (as glucosamine), 3·2 p.c. nitrogen, and 0·06 p.c. phosphorus. Hexosamine was determined by Procedure A (Svennerholm, 1956-1957), free sialic acid was estimated by the method of Warren (1959) and other methods of analyses were those used previously (North and Doery, 1961).

Albumin. Crystalline preparations of bovine and human plasma albumin were supplied by Mr. C. Guthrie of these Laboratories.

Neuraminidase. Dr. G. Ada of the Walter and Eliza Hall Institute of Medical Research supplied generous samples of crystalline neuraminidase.

Staphylococcal toxin.

A preparation similar to that used in previous work (North and Doery, 1960a; Doery and North, 1960) was used throughout. The LD_{50} I.V. for mice weighing 20 gm. was 1 ml. of 1:40 dilution in isotonic saline (0·85 p.c.).

Experimental animals. White mice weighing 16-18 gm. were used throughout.

Inactivation of staphylococcal toxin by ganglioside.

For inactivation studies, unless stated otherwise, heated ganglioside was used, i.e. ganglioside (1-10 mg./ml. H_2O) held in a stoppered tube in a water bath at 80° C. for 10 min., then cooled immediately to room temperature. Subsequent dilutions were made in isotonic saline. Toxin and ganglioside were held together at 37° C. under the conditions described for each experiment and injected intravenously into mice. Deaths were recorded up to seven days.

Ultracentrifuge analysis.

A Model E Spinco ultracentrifuge was used with standard 12 m.m. cells and wedge windows, speed 42,040 and 52,640 r.p.m., 20° C.

Effect of heat on ganglioside.

An aqueous solution of ganglioside (10 mg./ml.) was prepared and portions were heated in stoppered pyrex tubes in water baths at 37° C., 80° C. and 100° C. for various times, then cooled immediately to room temperature. Aliquots of the unheated and heated solutions were removed for the estimation of free sialic acid and for inactivation tests. The remainder of each solution was then submitted to ultracentrifuge analysis after adding NaCl to give a concentration of 0·2 M in each case.

Action of neuraminidase on ganglioside.

This was studied under conditions similar to those described by Ada, French and Lind (1961) for the action of neuraminidase on sialyl lactose at pH 5·6 in 0·1 M tris-maleate buffer with and without the addition of both calcium chloride and bovine plasma albumin.

Inactivation of staphylococcal toxin by ganglioside previously treated with neuraminidase.

The enzyme substrate mixture, neuraminidase (0·4 μg./ml.) and ganglioside (2 mg./ml.) in 0·1 M tris-maleate buffer at pH 5·6, was held at 37° C. for 1-23 hr. As a control, ganglioside was treated in a similar way with neuraminidase which had been inactivated previously by holding 1 ml. (1 μg./ml. in 0·1 M tris-maleate buffer pH 5·6) in a water bath at 100° C. for 5-10 min. At intervals portions of each ganglioside solution were removed for the estimation of free sialic acid. Other portions were diluted appropriately with saline and held at 4° C. until tested for their interaction with staphylococcal toxin.

RESULTS.

Effect of human plasma albumin on the inactivation of staphylococcal toxin by ganglioside.

It was reported earlier that ganglioside failed to inactivate toxin in the presence of human plasma albumin (North and Doery, 1961). This observation

TABLE 1.

Effect of holding ganglioside and albumin together at 37° C. prior to inactivation of staphylococcal toxin.

Group	Material injected	Concentration of albumin injected mg./ml.	Survivors 7 days
1	Toxin alone	—	2/12
2	Toxin+ganglioside (0·05 mg./ml.)	—	12/12
3	Toxin+ganglioside (0·05 mg./ml.) + albumin	0·08	1/6
4	Toxin+ganglioside (0·05 mg./ml.) + albumin	0·025	1/6
5	Toxin+ganglioside (0·05 mg./ml.) + albumin	0·008	1/6
6	Toxin+ganglioside (0·05 mg./ml.) + albumin	0·0025	3/6
7	Toxin+ganglioside (0·05 mg./ml.) + albumin	0·0008	2/6
8	Toxin+ganglioside (0·05 mg./ml.) + albumin	0·00025	6/6

Toxin alone, and toxin+ganglioside held at 37° C. for 1 hr. (Groups 1 and 2). Ganglioside and albumin held together at 37° C. for 1 hr. then added to toxin and held for a further hour at 37° C. (Group 3). 0·5 ml. of each preparation injected into mice I.V.

TABLE 2.

Effect of albumin on the inactivation of staphylococcal toxin by ganglioside.

Group	Treatment of material injected	Concentration of ganglioside and albumin injected (mg./ml.)		Survivors 7 days
		Ganglioside	Albumin	
1	Toxin alone—37° C.—1 hr.	—	—	0/10
2	Toxin and ganglioside held together at 37° C. for 1 hr.	0·05	—	5/5
		0·075		5/5
		0·11		5/5
3	Albumin and ganglioside held at 37° C. for 30 min. then added to toxin, and mixture held at 37° C. for further 30 min.	0·075	0·016	1/5
		0·075	0·05	0/5
		0·075	0·16	1/5
4	Albumin and ganglioside held at 37° C. for 30 min. then added to toxin, and mixture held at 37° C. for further 30 min.	0·11	0·016	0/5
		0·11	0·05	1/5
		0·11	0·16	0/5
5	Toxin and ganglioside held at 37° C. for 30 min. then added to albumin, and mixture held at 37° C. for further 30 min.	0·075	0·016	5/5
		0·075	0·05	5/5
		0·075	0·16	5/5
6	Toxin and ganglioside held at 37° C. for 30 min. then added to albumin, and mixture held at 37° C. for further 30 min.	0·11	0·016	5/5
		0·11	0·05	5/5
		0·11	0·16	5/5

0·5 ml. of each preparation injected into mice I.V.

was extended in the present study and Table 1 shows the effect of holding a constant amount of ganglioside with decreasing amounts of albumin at 37° C. for 1 hr. prior to mixing with staphylococcal toxin. It can be seen that 0·025 mg. of ganglioside in a dose of 0·5 ml. completely inactivated the challenge dose of toxin. Inactivation was prevented by prior contact of this amount of ganglioside with 0·004 mg. of albumin at 37° C. A detectable effect was produced by as little as 0·0004 mg. while 0·00012 mg. of albumin failed to affect the inactivation of toxin by ganglioside. In independent controls albumin did not affect the activity of the toxin.

The possibility of reversing the interaction of either toxin or albumin with ganglioside was considered. Table 2 shows the results of an experiment designed to test this point. The failure of mice in Groups 3 and 4 to survive confirmed the results of the previous experiment, i.e. that toxin did not reverse the effect of prior contact of ganglioside with albumin. The complete protection of

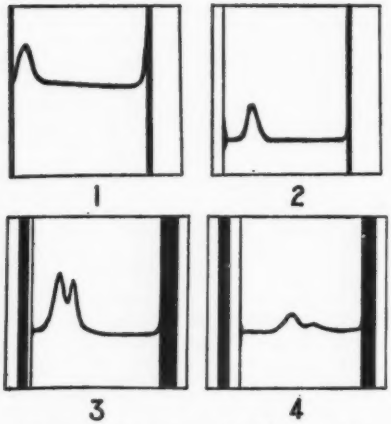

Fig. 1. Diagrams of schlieren photographs (angle 60°) of albumin and ganglioside and mixtures of these. All samples were held at 37° C. for 1 hr. in 0·85 p.c. NaCl and centrifuged in this buffer. Photographs taken 40 min. after attaining maximum speed.
1 = albumin 0·5 p.c., $s_{20\ buffer} = 4·3$.
2 = ganglioside 0·5 p.c., $s_{20\ buffer} = 9·8$.
3 = albumin 0·5 p.c. + ganglioside 0·5 p.c., $s_{20\ buffer} = 6·2$, 9·4.
4 = albumin 0·1 p.c. + ganglioside 0·5 p.c., $s_{20\ buffer} = 10·9$, 14·5.
Samples 1 and 2 speed = 42,040 r.p.m.
Samples 3 and 4 speed = 52,640 r.p.m.
Sedimentation, left to right.

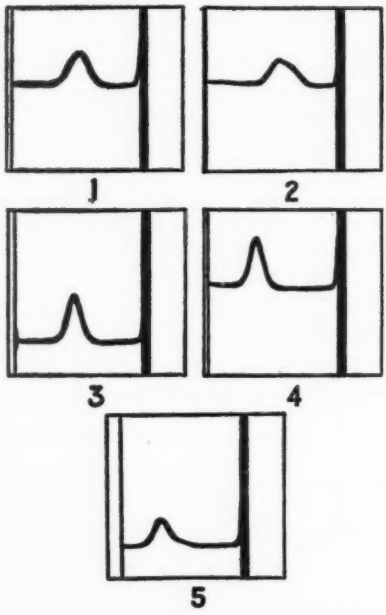

Fig. 2. Diagrams of schlieren photographs (angle 60°) of ganglioside (1 p.c. in 0·2 M NaCl) preparations 1-5 in Table 4, taken at times stated after attaining maximun speed (42,040 r.p.m.). 1 = 88 min.; 2 and 3 = 120 min.; 4 and 5 = 104 min. Sedimentation, left to right.

mice in Groups 5 and 6 shows that 0·008-0·08 mg. of albumin did not reverse the inactivation of toxin by 0·037-0·055 mg. of ganglioside (dose 0·5 ml.), the higher level of albumin being 20 times the minimum amount which prevented the subsequent inactivation of toxin by 0·025 mg. of ganglioside (Table 1). The serum globulins (γ, a_1 and a_2) interfered with the interaction of staphylococcal toxin and ganglioside in a similar manner.

Ultracentrifuge analysis of ganglioside, albumin and mixtures of these.

Fig. 1 shows the results of the ultracentrifuge analysis of isotonic saline solutions of ganglioside, albumin and mixtures of these after holding at 37° C. for 1 hr. The increase in the $s_{20\ buffer}$ value of the albumin component in the 0·5 p.c. albumin, 0·5 p.c. ganglioside mixture and the appearance of the peak with $s_{20\ buffer}$ value of 14·5 in the 0·1 p.c. albumin, 0·5 p.c. ganglioside mixture indicate that interaction has occurred. The same trend was observed with other ganglioside preparations.

Effect of heat treatment of aqueous solutions of ganglioside on the inactivation of staphylococcal toxin.

All experiments confirmed our earlier observation that ganglioside inactivated staphylococcal toxin more effectively if aqueous solutions of ganglioside were held in a water bath at 100° C. prior to interaction with staphylococcal toxin.

TABLE 3.

Inactivation of staphylococcal toxin by ganglioside unheated and heated at 10 mg./ml. H_2O.

Material injected	Concentration of ganglioside injected mg./ml.	Day of death	Survivors 7 days
Toxin alone	—	1, 1, 1, 1, 2, 2, 2, 2, 3	1/10
Toxin + ganglioside (unheated)	0·11	1, 1, 1, 1	1/5
	0·17	1, 1	3/5
	0·25		5/5
Toxin + ganglioside (10 min. 37° C.)	0·075	1, 1, 1, 1, 2	0/5
	0·11	1, 1, 2, 2	1/5
	0·17	1, 2, 2	2/5
Toxin + ganglioside (10 min. 80° C.)	0·033	2, 5	3/5
	0·05		5/5
	0·075	5	4/5
Toxin + ganglioside (20 min. 80° C.)	0·033	1, 2	3/5
	0·05		5/5
	0·075		5/5
Toxin + ganglioside (30 min. 100° C.)	0·05	4	4/5
	0·075		5/5
	0·11		5/5

Ganglioside at 10 mg./ml. H_2O was heated for the times and at temperatures as shown, then diluted appropriately in isotonic saline. Toxin alone, and toxin + ganglioside were held at 37° C. for 1 hr. then 0·5 ml. injected into mice I.V.

Table 3 shows that the activity of ganglioside against staphylococcal toxin was not altered significantly by the heat treatment at 37° C. while 10 min. in the water bath at 80° C. increased its activity fivefold. This enhanced activity was maintained after 20 min. at this temperature and was possibly only slightly less after holding the ganglioside for 30 min. at 100° C. Samples which had been heated for 10 min. at 80° C. then held at 4° C. or frozen at −5° C. overnight were found to have activities intermediate between those of the heated and unheated solutions.

It is well known that sialic acid is readily released from ganglioside on heating aqueous solutions (Bogoch, 1958). From Table 4 it can be seen that

TABLE 4.

Sedimentation coefficients and free sialic acid contents of ganglioside unheated, and after heat treatment at 10 mg./ml. H_2O

Preparation	Treatment of ganglioside	Free sialic acid as p.c. ganglioside	s_{20} buffer
1	Unheated	0·0	10·7
2	10 min. at 37° C.	0·2	8·9
3	10 min. at 80° C.	2·7	7·0
4	20 min. at 80° C.	4·7	6·7
5	30 min. at 100° C.	18	5·6

Sedimentation coefficients were determined on 1 p.c. solutions in 0·2 M NaCl.

at 37° C. only 0·2 p.c. of the ganglioside was released as free sialic acid. With stronger heating, namely, 30 min. at 100° C., the sialic acid released increased up to 18 p.c. of the weight of the ganglioside. N-Acetyl neuraminic acid alone has no detectable action on staphylococcal toxin. This was further borne out by the fact that the solution remaining after the removal of the free sialic acid from the heated preparations, by dialysis, was equally as active as the undialysed preparation. However, freeze drying the product remaining after dialysis reduced its activity significantly.

Gangliosides are known to occur in large aggregates of the basic unit (Bogoch, 1958). It had occurred to us that the altered activity shown by ganglioside after various treatments, including heating, may have been associated with a change in the size of the aggregate, and ultracentrifuge analyses were carried out to test this point. The data obtained are shown in Fig. 2 and Table 4. Fig. 2 shows the change in the sedimentation pattern at comparable times, and from the sedimentation coefficients it can be seen that the rate of sedimentation falls with increasing severity of heat treatment (Table 4).

Action of neuraminidase on ganglioside.

The action of this enzyme was studied because it was of interest to examine the interaction of toxin and ganglioside after sialic acid had been removed from the latter by means less severe than heat treatment. Ada, French and

Lind (1961) found optimum release of sialic acid from sialyl lactose by neuraminidase in 0·1 M tris maleate buffer which contained 0·05 p.c. bovine plasma albumin and 0·001 M $CaCl_2$.

Since albumin interacted with ganglioside and the addition of calcium ions interfered with the interaction of ganglioside and staphylococcal toxin (unpublished data) conditions for the release of sialic acid from ganglioside by neuraminidase in the absence of both albumin and calcium chloride were studied. Bovine plasma albumin (0·03 p.c.) increased the rate of release of sialic acid strikingly, whereas calcium chloride (0·1-0·0001 M) had little effect.

TABLE 5.

Effect of 0·03 p.c. (w/v) albumin on the release of sialic acid from ganglioside at 2 mg./ml. in 0·1 M tris maleate buffer, pH 5·6 by neuraminidase at 37° C.

Concentration of enzyme—μg./ml.	Addition to albumin	Free sialic acid as p.c. ganglioside in 1 hr.
0·5	—	14·5
0·4	—	8·5
0·016	+	9·3

Table 5 shows that in the absence of albumin it was necessary to increase the concentration of neuraminidase > twentyfold, i.e. from 0·016 to 0·4 μg./ml. to release the same amount of sialic acid in 1 hr. as that released in the presence of 0·03 p.c. albumin. Table 6 shows that calcium chloride (0·001 M) only slightly increased the rate of release of sialic acid irrespective of the addition of albumin to the reaction mixture. The maximum amount of sialic acid released under optimum conditions was never more than 18 p.c. of the weight of the ganglioside, i.e. 62 p.c. of the total sialic acid. Failure to release additional sialic acid may have been due to inactivation of the enzyme on pro-

TABLE 6.

Effect of calcium chloride (0·001 M) and albumin (0·05 p.c.) on the release of sialic acid from ganglioside (2 mg./ml.) by neuraminidase (0·015 μg./ml.) in 0·1 M tris maleate buffer, pH 5·6, at 37° C.

Time of reaction	Free sialic acid as p.c. ganglioside			
	Albumin nil		Albumin 0·05 p.c.	
	$CaCl_2$-nil	$CaCl_2$ 0·001 M	$CaCl_2$-nil	$CaCl_2$ 0·001 M
10 min.	3·4	4·0	4	5
20 min.	—	4·4	6·5	9
1 hr.	4	5	10	13
4½ hr.	3	5·3	—	—
5½ hr.	—	—	16	17

longed incubation. The addition of fresh enzyme was not included to test this point. Rosenberg, Binnie and Chargaff (1960) recently re-examined the action of neuraminidase on brain mucolipid using an enzyme preparation with slightly different properties from that used here and found that 66-72 p.c. of the total sialic acid was liberated.

Inactivation of staphylococcal toxin by ganglioside after treatment with neuraminidase.

No significant difference was found in the sialic acid released from either heated or unheated ganglioside by neuraminidase, therefore the more active preparation, i.e. heated ganglioside, was used for the toxin inactivation tests.

Based on the experiments described above, the conditions chosen for the enzyme treatment of ganglioside were those shown in Table 7 where the free

TABLE 7.

Free sialic acid released from ganglioside (2 mg./ml.) by neuraminidase (0·4 μg./ml.) in 0·1 M tris maleate buffer, pH 5·6, at 37° C.

Enzyme treatment		Free sialic acid as p.c. ganglioside
Time	Activity	
1 hr.	Active	8·5
1 hr.	Inactive	2·2
4½ hr.	Active	16
4½ hr.	Inactive	2·3
23 hr.	Active	17
23 hr.	Inactive	2·5

sialic acid is recorded as a percentage of the ganglioside after 1, 4½ and 23 hr. treatment respectively, with both active and inactive neuraminidase. The free sialic acid (2·2-2·5 p.c.) found in ganglioside treated with inactivated enzyme is of the order of that released in the initial heating of the ganglioside.

Table 8 shows the results of two separate inactivation experiments carried out on separate ganglioside preparations. In the first of these all the mice received a dose of toxin which was slightly more than an LD_{50} and ganglioside was tested after 1 hr. treatment with neuraminidase. It will be seen that ganglioside treated with either active or inactive neuraminidase was equally as effective as untreated ganglioside in inactivating staphylococcal toxin. Thus, the release of 8·5 p.c. of the ganglioside as free sialic acid by neuraminidase did not affect the interaction with staphylococcal toxin. In the second experiment the mice received at least one LD_{100} of toxin. It will be seen that all groups of mice receiving enzyme-treated ganglioside fared worse than those receiving untreated ganglioside. However, there was no significant difference in the inactivation of toxin by ganglioside treated with either active or inactive

TABLE 8.

Inactivation of staphylococcal toxin by ganglioside treated with neuraminidase under conditions shown in Table 7.

Expt.	Group	Material injected	Enzyme treatment of ganglioside		Concentration of ganglioside injected mg./ml.	Day of death after challenge	Survivors	
			Activity	Time hr.				
1	1	Toxin alone	—	—	—	1, 1, 1, 1, 2	3/8	
	2	Toxin + ganglioside	—	—	0·033	1, 2	3/5	
					0·05		5/5	
					0·075		5/5	
	3	Toxin + ganglioside	Active	1	0·033	1	4/5	
					0·05		5/5	
					0·075		5/5	
	4	Toxin + ganglioside	Inactive	1	0·05		5/5	
					0·075		5/5	
					0·11		5/5	
2	1	Toxin alone	—	—	—	h, 1, 1, 1, 1	0/5	
	2	Toxin + ganglioside	—	—	0·033	1, 1, 6	2/5	12/15
					0·05		5/5	
					0·075		5/5	
	3	Toxin + ganglioside	Active	4½	0·033	h, 1, 1, 1	1/5	9/15
					0·05	h, 1	3/5	
					0·075		5/5	
	4	Toxin + ganglioside	Inactive	4½	0·033	h, h, 1, 1, 1	0/5	6/15
					0·05	1, 1, 1	2/5	
					0·075	1	4/5	
	5	Toxin + ganglioside	Active	23	0·033	1, 1, 1, 1, 2	0/5	9/15
					0·05	—	5/5	
					0·075	2	4/5	
					0·11	7	4/5	
					0·17		5/5	
	6	Toxin + ganglioside	Inactive	23	0·033	h, 1, 1	2/5	9/15
					0·05	1, 1	3/5	
					0·075	1	4/5	
					0·11		5/5	

For the inactivation of the toxin, equal volumes of toxin and ganglioside, treated as shown, were mixed and held together at 37° C. for 1 hr., then 0·5 ml. injected into mice I.V.

enzyme for 4½ or 23 hr. Thus, after the release of 8·5, 16 and 17 p.c. of ganglioside as sialic acid (Table 7), ganglioside preparations were equally as effective in inactivating staphylococcal toxin (Table 8). The ganglioside preparations treated with enzyme for 4½ hr. were the only samples which were held overnight at 4° C. prior to the inactivation tests. Other inactivation tests followed the enzyme treatment within 1 hr. In the second test the lower activity of all enzyme-treated ganglioside solutions can probably be attributed to the longer time in the buffer system.

DISCUSSION.

The work described above confirmed our belief that proteins other than toxins may interact with ganglioside. If we assume that gangliosides combine with toxins and serum proteins, such combination is firm in the case of staphylococcal toxin, and neither toxin nor albumin displaced the other from ganglioside after interaction at 37° C. However, the upper limit of concentration of the displacing agent may not have been reached in all cases. It had been shown in previous experiments (North and Doery, 1961) that toxin was inactivated by gangliosides in mixtures containing either peptone or gelatin. The peptone (0·5 p.c.) caused some reduction in the inactivation of one LD_{100} of staphylococcal toxin by ganglioside after 35 minutes' contact at 37° C. whereas after 5 hours, inactivation was complete. A dose of tetanus toxin containing at least 10 LD was still inactivated by ganglioside in the presence of 0·1 p.c. gelatin. The results of these experiments and the failure to inactivate the neurotoxin of *Shigella shigae* with ganglioside (North, Pawlyszyn and Doery, 1961) indicate that a range of affinities exists in the interaction of gangliosides and proteins.

The experiments with ganglioside in which sialic acid was removed by either heat, or less severely by neuraminidase, show that 62 p.c. of the sialic acid may be removed without destroying the activity of the ganglioside on staphylococcal toxin. Thus, all the sialic acid units of ganglioside are not vital sites in this reaction. The ultracentrifuge analysis provided other information which seems relevant to the understanding of the effect of heat on ganglioside. The decrease in the $s_{20\ buffer}$ value of the major component of the ganglioside with increasing severity of heat treatment (Table 4) is consistent with either a disaggregation to smaller units, or to an unfolding leading to a molecule with higher frictional coefficient than the original. Either of these possibilities supports the idea that the increased interaction between heated ganglioside and toxin is due to an increase in accessibility of active sites on the ganglioside. The loss of 62 p.c. of the sialic acid on heating does not, of course, preclude the remaining sialic acid from being the point of interaction. In the structures proposed for ganglioside by Klenk and Lauenstein (1953) and Bogoch (1958) fatty acids and sialic acid both occupy end positions. Sialic acid has been shown to play an important rôle in many of the compounds in which it is found

but the significance of fatty acids in the combination of ganglioside and toxin must not be overlooked in view of the strong combinations that occur between long chain fatty acids and proteins.

The findings of McIlwain (1961) which became available while this paper was being prepared are of particular interest. In studying the respiratory response of cerebral tissues to applied electrical pulses he showed that the response may be inhibited by basic proteins (protamines, histones) or a synthetic poly l-lysine and restored by ganglioside. In this case the sialic acid units in the ganglioside were considered to be the active sites, and complex formation between these and the positive charges of the protein was regarded as the basis of this interaction. In discussing the interaction of tetanus toxin and ganglioside shown by van Heyningen (1959), McIlwain (1961) suggested that a specific structural relationship might be involved. In view of the range of toxins now known to interact with ganglioside, it seems unlikely that such a relationship would occur in all cases. Different degrees of affinity between gangliosides and toxins may exist, and indeed have been demonstrated, but quantitative studies are limited by the availability of pure toxin and a standard preparation of ganglioside.

Ganglioside has been prepared by a number of methods (van Heyningen, 1959, and McIlwain, 1961) and the first step in that prepared here involved extraction of grey matter with methanol at 50°-60° C. Preparations which cannot be distinguished on the basis of chemical composition, while all active, have shown detectable differences in activity towards staphylococcal toxin. Preliminary experiments suggest that the incorporation of cations in ganglioside may modify this activity, while it has been proposed that disaggregation or unfolding of ganglioside units is responsible for the increase in the activity of heated preparations. Ganglioside prepared by different methods may well vary in size of aggregate and hence biological activity. However, these *in vitro* studies have yet to be translated into the details of *in vivo* mechanisms for which a general hypothesis for the release of ganglioside at the site of contact with toxin has been made (North and Doery, 1960b, 1961). In this regard McIlwain and Balakrishnan (1961) have now ascribed an *in vivo* rôle for ganglioside in an ion transport mechanism which extends the earlier observation of McIlwain (1961) on the interaction of basic proteins and ganglioside.

Acknowledgments. We are indebted to Dr. G. L. Ada for the gift of crystalline neuraminidase and to Mr G. Wilson and Miss Frances Bates for the ultracentrifuge data. We thank Miss Joan Pearson, Miss Edith Russell and Miss Sigrid Marklein for invaluable help in many ways. Discussions with Mr. I. J. O'Donnell, Dr. K. Turner and Mr. E. R. B. Graham were most helpful.

We regret that the interesting papers of Dr. van Heyningen and his associates (W. E. van Heyningen and P. A. Miller, 1961, J. gen. Microbiol., 24, p. 107, and A. W. Bernheimer and W. E. van Heyningen, *ibid.*, p. 121) arrived too late for adequate discussion in the text. Further studies are needed to determine the quantitative relations between the fixation and inactivation of bacterial toxins by ganglioside as well as their relative significance *in vivo*.

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THE INTERCONVERSION OF OESTRONE AND OESTRADIOL-17β BY HUMAN AND RAT BLOOD

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SUMMARY.

The interconversion of oestrone and oestradiol-17β occurs in human and rat blood cells and to a lesser extent in plasma. Reduction of oestrone produces only oestradiol-17β which has been identified by preparation of its pipsylate. This can be separated from the pipsylate of oestradiol-17α by paper chromatography. Oestrone and oestradiol-17β also yield polar substances when incubated with blood preparations.

The preparation and constants of a number of oestrogen pipsylates are described.

INTRODUCTION.

The interconversion of oestrone and oestradiol-17β by various preparations has been described by several workers. The kinetics of this conversion by yeast has been studied by Portius and Repke (1957) while the kinetics and specificity of a 17β-hydroxysteroid dehydrogenase in human placenta were investigated by Hollander *et al.* (1958) and by Langer *et al.* (1959).

The presence of very small amounts of oestradiol-17β in circulating rat blood after infusion of oestrone-C^{14} has been reported (Wotiz *et al.*, 1958). The metabolism of oestradiol-17β and the 16,17-oxygenated oestrogens has been studied in human liver slices and human blood cells by Breuer (1959) and in rat, cattle and human erythrocytes by Portius and Repke (1960a, b, c). Differences in the hydroxysteroid dehydrogenase activities of these species were observed. The metabolism of oestrone by bovine blood has been studied by Axelrod and Werthessen (1960): blood obtained from young steers and bulls apparently did not metabolise oestrone but pregnant cow blood converted oestrone to oestradiol-17α, which was isolated and characterised. No conversion to oestradiol-17β could be observed.

In the present communication we describe the interconversion of oestrone and oestradiol-17β catalysed by rat and human blood cells and plasma. We have also observed a species difference in the conversion of oestrone to the 17β-hydroxy compound and have been able to separate the 17α and the 17β oestradiols by chromatography of their p-iodobenzenesulphonyl (pipsyl) derivatives prepared by a method similar to that of Leegwater (1956). After incu-

bation of oestrone with human or rat blood oestradiol-17β was the only reduction product detected.

As the pipsyl derivatives of oestrogen metabolites may be useful for identification in metabolic studies the preparation and chromatography of a number of these compounds are also described.

EXPERIMENTAL.

Steroids.

Oestrone-16-C^{14} (activity 4 mc. per mg.) and S^{35}-pipsylchloride were obtained from U.K.A.E.A., Amersham, U.K., and oestradiol-17β-2-C^{14} (activity 1·5 mc. per mg.) from N.E. Nuclear Corporation, Boston, U.S.A. Before use the compounds were chromatographed in solvent system 1 (see below) and the areas corresponding to oestrone and oestradiol-17β were eluted with methanol.

Preparation of human and rat blood incubation media.

Heparinized rat or human blood (7 ml., containing 500 I.U. heparin/ml. blood) was centrifuged at 3000 r.p.m. The plasma was removed and retained for use in the experiment. The blood cells were washed twice with a buffer solution of the following composition: NaCl, 113 mM.; KCl, 36 mM.; $CaCl_2$, 2 mM.; Na_2HPO_4, 4 mM.; KH_2PO_4, 1·2 mM.; glucose, 5 mM.; pH, 7·4.

Solutions of the oestrogens in methanol were evaporated to dryness under nitrogen to deposit either oestrone (4 μg, 80,000 c.p.m.) or oestradiol-17β (4 μg, 35,000 c.p.m.) on the walls of the incubation tubes. Incubations with blood cells, plasma and water were carried out simultaneously as follows:

Tube 1: Oestrogen with 3 ml. of washed blood cells and 1 ml. of the above buffer solution.
Tube 2: Oestrogen with 3 ml. of plasma and 1 ml. of buffer.
Tube 3: Oestrogen with 3 ml. of water and 1 ml. of buffer.

The liquid in each tube was saturated with oxygen at 37° C. and the incubation was carried out in unstoppered tubes for two hours. A sufficient amount of oxygen was bubbled through the liquid in each tube during the incubation to maintain saturation and agitate the liquid.

Extraction.

After incubation the contents of each tube were immediately added to 100 ml. water and the mixture continuously extracted with ether for 12 hours. To determine whether radioactive oestrogen was still adhering to the walls of the incubation tubes the latter were washed with hot methanol and the resulting solutions assayed for radioactivity. Less than 0·5 p.c. of the added radioactivity could be detected in any of these washings.

Paper chromatography and detection of radioactivity.

The ether extract from the incubation mixture was evaporated to a small volume and chromatographed on Whatman No. 1 paper using the organic layer of toluene 15, iso-octane 5, methanol 16, water 4, as the developing solvent (chromatography system 1) (Brown *et al.*, 1958). Radioactive areas on the paper chromatograms were detected in a windowless gas-flow (argon, ethanol quenched) chromatogram scanner and an approximate measure of the radioactivity present could be obtained from the area under the peak. For more accurate determinations the radioactive areas were eluted with methanol and the activity determined in a windowless gas-flow (argon, ethanol) counter. Non-radioactive oestrogens used as controls in the paper chromatography were detected using $FeCl_3$-$K_3Fe(CN)_6$ reagent (Barton *et al.*, 1952).

Oestrogen pipsylates were chromatographed using the organic phase of the system

iso-octane 10, methanol 8, water 2, as the developing solvent (chromatography system 2). Non-radioactive oestrogen pipsylates were detected using an $SbCl_3$ reagent (Neher and Wettstein, 1951).

The preparation of oestrogen pipsylates.

Oestrogen (50 mg.) was dissolved in 5 ml. acetone and 5 ml. 2 p.c. sodium tetraborate solution added. To this solution 1 to 3 mole equivalents of pipsyl chloride were added and the solution kept at room temperature for 15 minutes. After the addition of a further 5 ml. 2 p.c. sodium tetraborate the solution was heated to 50° C. for 30 minutes. The reaction mixture was then partitioned between benzene and N NaOH, the benzene layer washed with a little water and dried over sodium sulphate. The pipsylates were obtained by evaporation of the benzene solution and were recrystallised from methanol.

The preparation of pipsylates of oestrogen metabolites.

The radioactive areas of the chromatograms were eluted with warm methanol in a small soxhlet extractor for 3 hours. The methanol was removed at 40° C. under nitrogen, the residue dissolved in 1 ml. of acetone and 0·3 ml. of 2 p.c. sodium tetraborate solution was added followed by 0·5 mg. of pipsyl chloride in acetone. After 15 minutes at room temperature a further 0·1 ml. 2 p.c. sodium tetraborate was added and the solution then heated to 50° C. for 30 minutes. The solution was transferred to a separating funnel containing 10 ml. benzene and 5 ml. N sodium hydroxide and was washed in with a further 10 ml. benzene, 10 ml. water and 10 ml. benzene in that order. After shaking the aqueous layer was discarded. The benzene layer was washed with three 10 ml. volumes of water, evaporated to a small volume and chromatographed on chromatography system 2.

The preparation of S^{35}-pipsylated oestrogens.

Approximately 1 mole equivalent of S^{35}-pipsyl chloride was added to 2 mg. of oestrogen dissolved in 5 ml. of acetone and 0·3 ml. of 2 p.c. sodium tetraborate solution. This solution was then heated on a water bath at 50° C. for 30 minutes and the pipsylate extracted as described above. The final benzene layer was evaporated to 10 ml. and aliquots used for chromatography as required.

RESULTS.

In Fig. 1 scans of the chromatograms obtained from the ether extracts obtained after incubation of oestrone with rat blood cells, oestradiol-17β with human blood cells and oestrone with human plasma are recorded. The presence of oestrone, oestradiol-17β and other radioactive material can be observed.

Each chromatogram was divided into three sections corresponding to the oestrone area, the oestradiol-17β area and the remainder of the chromatogram and the radioactivity eluted therefrom with methanol. In Table 1 the counts recovered in each area are expressed as a percentage of the combined counts eluted from these three areas and each result shown represents the mean value of eight experiments. The total counts eluted from the paper chromatogram represented between 80 and 90 p.c. of the counts added initially to the incubation tubes.

In order to determine whether the oestradiol produced from oestrone on incubation was the 17α or the 17β isomer, the eluates from the metabolite areas were pipsylated and run with chromatography system 2.

Fig. 2(a) is the scan of oestradiol-17β pipsylate-S^{35} prepared using S^{35}-

pipsyl chloride and Fig. (2c) shows the separation of non-radioactive oestradiol-17α pipsylate and oestradiol-17β pipsylate (compounds detected with $SbCl_3$ reagent). It is apparent that only oestradiol-17β is formed.

TABLE 1.

*Percentage conversion of oestradiol-17β and oestrone with human and rat blood preparations**

Substrate	Blood fraction	Oestrone detected	Oestradiol-17β detected	Remaining radioactivity
Oestradiol-17β	Rat blood plasma	4·6%	84%	11·4%
	Rat blood cells	9·1%	79%	11·9%
Oestradiol-17β	Human blood plasma	6·4%	80%	13·6%
	Human blood cells	8·3%	79%	12·7%
Oestrone	Rat blood plasma	78%	2%	20%
	Rat blood cells	35%	54%	11%
Oestrone	Human blood plasma	82%	5·2%	12·8%
	Human blood cells	72%	14·2%	13·8%

* Results represent the mean of eight experiments and each fraction is expressed as a percentage of the total eluted radioactivity.

TABLE 2.

Pipsylate of:	(a) m.p.° C.	(b) $[\alpha]_D$	(c) R_f	Analyses (d) Theoretical	Found
Oestrone	179-181	+156°	0·87	C, 53·77	53·87%
				H, 4·85	4·82%
Oestradiol-17β	175-176	+121°	0·45	C, 53·53	53·86%
				H, 5·05	5·22%
Oestradiol-17α	80-82	+ 63°	0·56	C, 53·53	54·31%
				H, 5·05	5·67%
Oestriol	173-174	+165°	0·58	C, 52·01	52·37%
				H, 4·91	5·16%
16α-Hydroxyoestrone	167-170	+ 78°	0·80	C, 52·18	52·21%
				H, 4·58	4·83%
16β-Hydroxyoestrone	174-175	+ 90°	0·78	C, 52·18	52·37%
				H, 4·58	4·62%
17α-Ethinyloestradiol	78-80	+ 32°	0·42	C, 54·54	54·75%
				H, 5·22	5·29%

(a) Uncorrected.
(b) In chloroform; c ranged from 0·4 to 1·5.
(c) In solvent system iso-octane 10, methanol 8, water 2. Developing time 3½ hours at 19-21° C.
(d) Calculated for a monopipsylate.

Table 2 records the melting points, $[\alpha]_D$ values and Rf values in chromatography system 2 of seven oestrogen pipsylates. From the analytical figures it is apparent that only the phenolic hydroxyl group is pipsylated under the mild conditions of this reaction and hydroxyl groups at positions 16 and 17 are not affected. It is possible, however, that under more vigorous conditions these alcoholic hydroxyls would be pipsylated.

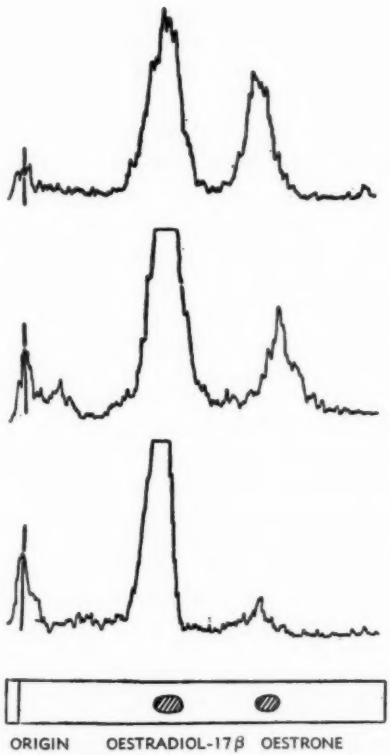

Fig. 1. Chromatogram scans of incubations of oestrogens with human and rat blood cells and plasma (chromatography system 1).
(a) Oestrone with rat blood cells.
(b) Oestradiol-17β with human blood cells.
(c) Oestradiol-17β with human plasma.

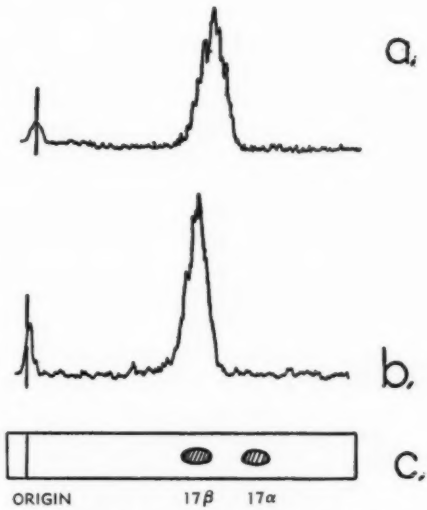

Fig. 2. Chromatogram scans of oestradiol-pipsylates (chromatography system 2, developing time 6 hours at 20° C.).
(a) Oestradiol pipsylate, labelled with C^{14}, isolated after incubation of oestrone with human blood cells.
(b) Oestradiol-17β pipsylate-S^{35}.
(c) Control non-radioactive oestradiol pipsylates.

DISCUSSION.

It can be seen from Table 1 that the major interconversion of oestrone and oestradiol-17β in blood occurs in the cellular fraction although small but significant quantities of metabolites could be detected after incubation with plasma. It is possible that the enzymes responsible for the interconversion in plasma originate from the cellular fraction and are present in the plasma only because of damage to the cells during fractionation. No haemolysis was observed during the preparation of the cells, but this does not necessarily indicate that leakage from the cells could not have occurred. The scans of chromatograms shown in Fig. 1 also indicate that the interconversion of oestrone and oestradiol-17β has occurred. In particular, a slight but distinct conversion of oestradiol-17β to oestrone by human plasma can be observed in Fig. 1 (c).

Table 1 also indicates that under similar experimental conditions a species difference exists in the conversion of oestrone by rat and human blood cells.

In the chromatograms shown in Fig. 1 radioactive material can be seen near the origin of the chromatograms, particularly in the products from the incubation of oestrone with human plasma. The material near the origin is apparently more polar than oestrone or oestradiol-17β and is probably a hydroxylated metabolite of one of these compounds.

We have been unable to find a paper chromatographic system which would separate oestradiol-17β from oestradiol-17α. Earlier investigators have either isolated the oestradiol and identified the particular isomer by its physical constants (Axelrod and Werthessen, 1960) or have utilised the differences in colour and spectra given by the two isomers in sulphuric acid (Umberger and Curtis, 1949). In the present experiments we have found that the pipsylates of oestradiol-17α and -17β can be separated by paper chromatography. From Fig. 2(a) it is apparent that the oestradiol produced on incubation of oestrone with human or rat blood preparations is the 17β isomer and that none of the 17α compound can be found. Both man and the rat are able to effect conversion of a 17-oxosteroid to a 17β-hydroxysteroid but not to the 17α epimer. This is in agreement with the results of Portius and Repke (1960a, b c), but must be contrasted with the metabolism in pregnant cow erythrocytes in which the 17α isomer is produced and not the 17β (Axelrod and Werthessen, 1960).

It is significant that the aqueous solutions remaining after the ether extraction contained only 0·5 to 1·0 p.c. of the initial radioactivity. This would suggest that water-soluble conjugates are not produced in this *in vitro* system although Jellinck (1960) has detected water-soluble, ether-insoluble products from oestrone incubated with rat liver slices.

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THE EFFECT OF THE ANTIOXIDANTS, BUTYLATED HYDROXY ANISOLE, BUTYLATED HYDROXY TOLUENE AND PROPYL GALLATE ON GROWTH, LIVER AND SERUM LIPIDS AND SERUM SODIUM LEVELS OF THE RAT[1]

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SUMMARY.

Confirmation of our previous finding that there are greater hazards in the use of butylated hydroxy toluene (BHT) than in that of butylated hydroxy anisole (BHA) as antioxidants for use in human foodstuffs was obtained. BHA and propyl gallate (PG) met one of the requirements for safety as a food additive. A sex difference in the effect of BHT was noted; BHT increased the relative weight of the male adrenal and had a greater deleterious effect on the growth rate of the male than of the female rat. A straight line relationship existed between the dietary level of BHT and the serum cholesterol level. When fed at the level of 0·1 p.c. of the diet, BHA increased the level of total serum cholesterol but no further elevations occurred at higher dosages. There was a relatively greater increase in the amount of serum free cholesterol than of ester cholesterol when BHA or BHT were fed. PG had no effect on the serum cholesterol levels. BHT increased both free and esterified adrenal cholesterol, a greater increase being noted in the latter. Although BHA and BHT produced increases in the size of the male adrenal none of the marked histological changes recorded by other workers using lethal doses of these antioxidants in the rabbit were apparent. The antioxidants had no effect on the concentration in the liver of the total and esterified cholesterol, total lipid or on the composition of the polyunsaturated fatty acids. However, as the liver weight was increased by BHA or BHT the absolute lipid content of the liver was increased.

INTRODUCTION.

It has been shown (Day, Johnson, O'Halloran and Schwartz, 1959) that the ingestion of the antioxidant butylated hydroxy toluene[4] (BHT) by rats elevates the serum cholesterol and phospholipid levels and in addition (Brown, Johnson

[1] This work was financed by the Department of the Army, as part of the research programme administered by the Army Food Science Establishment, Melbourne, Victoria, Australia.

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[4] Tenox BHT 2:6 di-t-butyl-p-cresol.

and O'Halloran, 1959) when BHT was fed in conjunction with a 20 p.c. lard supplement to the stock diet of the rat it did not conform to the generally accepted criteria (World Health Organisation, 1958) for safety as an additive to foodstuffs for human consumption. On the other hand, the antioxidant butylated hydroxy anisole[5] (BHA) in a 10 p.c. lard supplement conformed to these criteria.

Denz and Llaurado (1957) attributed the death of rabbits given large doses of BHA and BHT to an excessive loss of electrolytes, especially sodium, in the urine which results in increased urinary aldosterone excretion with depletion of lipid in the zona glomerulosa of the adrenal.

The present study was undertaken to compare the effects at different dietary levels in the rat of BHA, BHT and a third antioxidant, propyl gallate[6] (PG), in a 20 p.c. lard supplement to the stock diet on the growth, liver and adrenal weights, liver, adrenal and serum cholesterol levels, polyunsaturated fatty acid composition of the total liver lipid, serum sodium and histological appearance of the adrenal.

EXPERIMENTAL.

The stock diet, the method of preparation of the experimental diets and the general procedure for the care of the animals have been described previously (Johnson, O'Halloran and Hewgill, 1958). The stock diet in the present work was supplemented with 20 p.c. lard to which were added increasing amounts of antioxidant so that the diets contained 0, 0·1, 0·2, 0·3, 0·4, 0·5 p.c. (dry weight) antioxidant. The antioxidant was added at the expense of 0·5 p.c. cellulose in the control diet, i.e., the 0 p.c. antioxidant level.

Litters of the Norway Hooded strain of rat were culled so that the litters contained three males and three females. At weaning (28 days) a litter was randomly allocated to one of the dietary concentrations of BHT. Two litters were placed on each experimental diet, the animals being caged in pairs with the sexes segregated. This procedure was repeated with the two remaining antioxidants.

Six weeks later the rats were fasted overnight, anaesthetized with ether and blood was withdrawn by cardiac puncture. The animals were killed by further anaesthetization. The liver was removed, dried with filter paper, and weighed. The left adrenal was removed and weighed after any extraneous tissue had been dissected away. The right adrenal was fixed in 10 p.c. formol saline.

Ethanol-ether (3:1) extracts of the sera were made. The liver lipids were extracted with petroleum ether (BP 40-60° C.) in a Waring Blendor according to the method of Thompson, Ganguly and Kon (1949). The left adrenals of the males and females of one litter were separately pooled and extracted for two hours in a micro-soxhlet apparatus using a 5:1 ethanol-ether solvent.

The total cholesterol content of the lipid extracts was determined by the method of Zlatkis, Zak and Boyle (1953). The free cholesterol was precipitated as the digitonide which was centrifuged off, washed with acetone and analysed by the method of Zlatkis *et al.* (1953). The ester cholesterol was calculated by difference.

The total lipid content of the liver extracts was determined gravimetrically and the polyunsaturated fatty acid composition of the total liver lipids was determined by a modi-

[5] Tenox BHA approximately 85 p.c. 3-t-butyl-4-hydroxy anisole and 15 p.c. 2-t-butyl-4-hydroxy anisole.
[6] Naarden PG.

fication of the method of Davenport, Birch and Ryan (1956) using the potassium t-butoxide reagent.

Serum sodium was estimated by flame photometry. Some haemolysis occurred during the collection of the blood so that no attempt was made to estimate serum potassium.

Haemtoxylin-eosin and sudan IV staining were carried out on sections of the right adrenals.

RESULTS.

Weight and histological studies.

The effect of six weeks' feeding of the three antioxidants on the weight of the rat, expressed logarithmically, is shown in Fig. 1. The experimental design introduced a genetic effect which led to some variations between litters; despite this, as shown previously (Brown *et al.*, 1959), BHT significantly reduced the growth rate of the rats, especially the males. BHA and PG had no significant

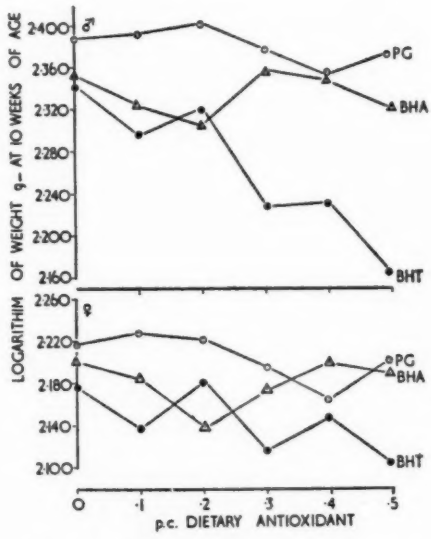

Fig. 1. The effect of six weeks' ingestion of a diet containing various levels of three antioxidants on the log. weight of rats placed on the diets at weaning.
1. Points shown are means of six observations.
2. L.S.D. ($p = 0{\cdot}05$) is the least significant difference for comparison of treatment means (error mean square on 24 degrees of freedom). BHT = 0·054, BHA = 0·036, PG = 0·029.

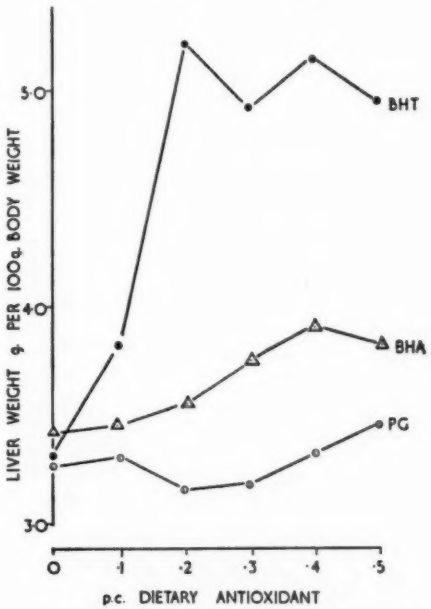

Fig. 2. The effect of three antioxidants on the relative liver weight of the rat.
1. Points shown are means of 12 observations, six male and six female.
2. L.S.D. ($p = 0{\cdot}05$). BHT = 0·444, BHA = 0·225, PG = 0·160.

effect on the growth rate of the animals even when 0·5 p.c. was present in the diet.

BHA and BHT produced increases in the liver weight-body weight ratio, while PG had no effect (Fig. 2). With BHT the relative increases were more marked and the absolute weight of the liver was increased.

BHA and BHT produced increases in the weight of the male left adrenal relatively to the body weight (Fig. 3) but had no consistent effect on the female left adrenal. PG had no effect on the relative weight of either the male or female adrenal.

No histological changes attributable to the treatments could be detected in the adrenal. There was no depletion of lipid staining of the zona glomerulosa or any increase in cytoplasmic basophilia as shown by Denz and Llaurado (1957) in the adrenals of rabbits given large doses of BHA or BHT.

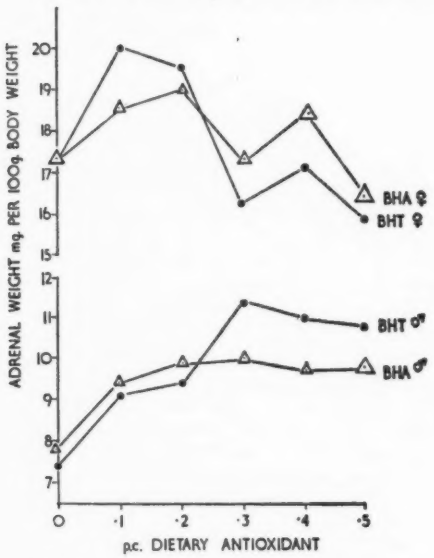

Fig. 3. The effect of BHA and BHT on the relative left adrenal weight of the rat.
1. Points are means of six observations.
2. L.S.D. ($p = 0{\cdot}05$). BHT = 3·37, BHA = 1·57.

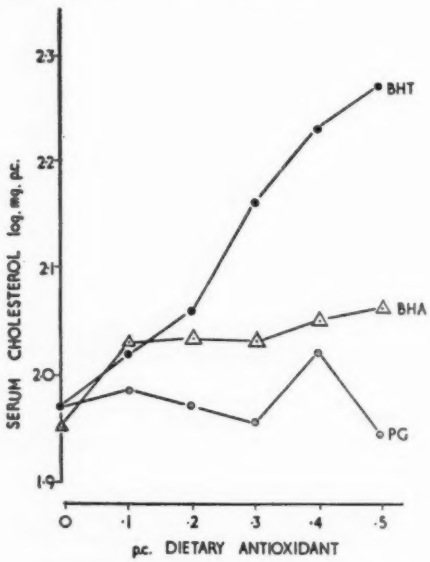

Fig. 4. The effect of three antioxidants on the log. serum cholesterol levels of the rat.
1. Points are means of 12 observations.
2. L.S.D. ($p = 0{\cdot}05$). BHT = 0·049, BHA = 0·041, PG = 0·039.

Chemical Determinations.

Sera.

The effect of the three antioxidants on the mean serum cholesterol levels, expressed logarithmically, of both male and female rats is shown in Fig. 4. The level of serum cholesterol was directly proportional to the level of dietary BHT.

The mathematical expression of the straight line response between these two variables was $y = 1{\cdot}9557 + 0{\cdot}6214x$. BHA also produced a significant increase in the level of serum cholesterol but this effect was not further increased significantly by increasing the dietary level of BHA above 0·1 p.c. PG did not have any significant effect on the serum cholesterol, with the exception of a diet containing 0·4 p.c. PG. This inconsistent effect was produced by the abnormally high level of serum cholesterol in three male rats of one litter.

The increase in total serum cholesterol produced by BHA and BHT was a result of increases in both the free and esterified fractions. However, as there was a greater increase in the free cholesterol fraction the percent. of esterified cholesterol in the total serum cholesterol fell (Fig. 5). PG had no effect on this value.

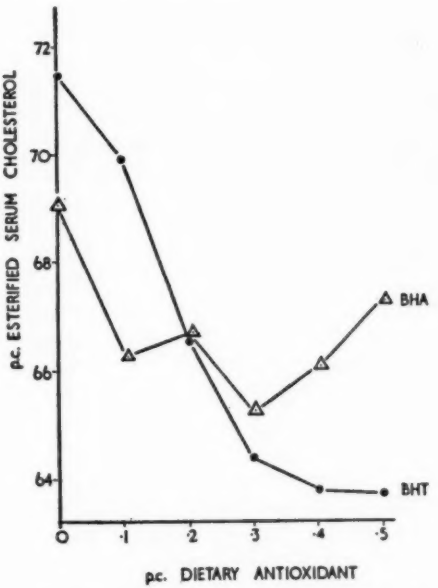

Fig. 5. The effect of BHT and BHA on the percent. esterified serum cholesterol.
1. Points are means of 12 observations.
2. L.S.D. ($p = 0{\cdot}05$). BHT = 2·5, BHA = 1·8.

The serum level of sodium was not affected by the inclusion of the antioxidants in the diet.

Adrenal cholesterol.

BHT produced a significant increase in the concentration of total adrenal cholesterol and there was a strong trend towards an increase in the percent. esterified adrenal cholesterol with increased dietary level of BHT (Figs. 6 and 7). BHA and PG had no significant effect on either of these constituents.

Liver lipids.

None of the antioxidants produced any significant changes in the concentration of the total or percent. esterified liver cholesterol, total liver lipid, or the $K^{0.1\ p.c.}_{1\ cm.}$ (extinction coefficient) value at the five wavelengths at which the polyunsaturated fatty acids are measured. However, as BHA and BHT produced increases in the relative weight of the liver, the absolute amounts of cholesterol and total lipid per rat liver were elevated.

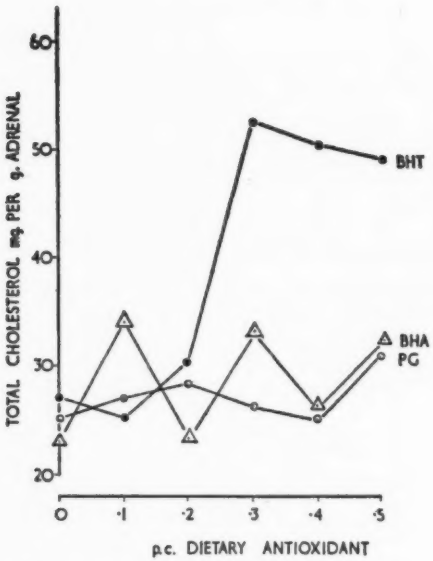

Fig. 6. The effect of three antioxidants on the concentration of total cholesterol of the rat left adrenal.
1. Points are means of four observations, being the pooled adrenals of three rats.
2. L.S.D. ($p = 0{\cdot}05$). BHT = 16·7, BHA = 27·2, PG = 10·9.

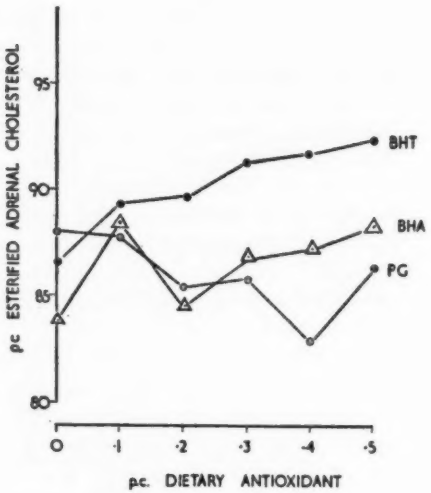

Fig. 7. The effect of three antioxidants on the percent. esterified adrenal cholesterol.
1. Points are means of four observations, being the pooled adrenals of three rats.
2. L.S.D. ($p = 0{\cdot}05$). BHT = 8·2, BHA = 8·3, PG = 5·3.

Urinary metabolites.

During food consumption studies the urine of the animals was absorbed on blotting paper placed in tin containers. The absorbed urine of the animals fed BHT and PG changed colour. That of the BHT animals turned bright pink and that of the PG animals turned purple. When the urine was collected in glass containers no such change occurred and these colours probably arose out of the metal catalysed oxidation of urinary metabolites of the two antioxidants (Dacre, 1961, and Booth *et al.*, 1959).

DISCUSSION.

The effects of BHA and BHT on the growth and liver weight of the rat confirm our previous finding (Brown *et al.*, 1959) that there is a greater hazard involved in using BHT as an additive for use in human foodstuffs than in using BHA. Neither BHA nor PG produced any significant deleterious effects on the growth rate of rats, giving confirmation to the work of Brown *et al.*

(1959), Wilder and Kraybill (1948), Graham *et al.* (1954), Graham and Grice (1955) and Orten, Kuyper and Smith (1948). BHA and PG thus fulfil one of the requirements for safety for use in human foods.

In accordance with our previous findings (Brown *et al.*, 1959) a sex difference in the effect of BHT was noted. BHT had a greater deleterious effect on the growth of the male than of the female rat and BHT and BHA produced increases in the relative adrenal weight of the male but not the female. Differences in the antioxidant potency of the male and female hormones have previously been observed: Meyer and McShan (1948) and Schuler and Meier (1956) showed that the oestrogens, in contradistinction to the androgens, can act as antioxidants, while Hood *et al.* (1950) showed that the fat from chickens given oestrogen implants was more stable than the fat from control animals.

The rat chronically fed a diet containing relatively less BHA or BHT than the acute doses (1 gm. per day) given to the rabbit by Denz and Llaurado (1957) did not show the marked histological changes of the adrenal produced in that species. The increases in the relative weight of the adrenals in the case of male rats fed BHA and BHT may well be a reflection of the changes produced by the dose level of the antioxidants which led to the death of the rabbits.

The straight line relationship between dietary BHT and the serum cholesterol is indicative of a cause and effect relationship and suggests the possibility that dietary levels of BHT normally ingested by man would cause similar but greatly reduced effects on the serum cholesterol. The straight line relationship has also enabled us to use the maximum concentration of BHT, i.e., 0·5 p.c. of the diet, in our later experiments and relate our findings to more physiological levels of dietary BHT. The lack of any further elevation of serum cholesterol on increasing the dietary level of BHA above 0·1 p.c. of the diet cannot be easily explained. It may be related to the relative antioxidant potency or ease of absorption of BHT and BHA. It is unlikely that the effects of BHA and BHT can be attributed solely to their antioxidant properties, as PG produced no changes in any of the parameters measured.

The elevated levels of serum cholesterol and the increase in the absolute amount of liver and adrenal cholesterol produced by BHA and BHT could be accounted for by an increased rate of synthesis or decreased breakdown of cholesterol or variations in absorption or excretion. Evidence that the first explanation is more likely will be presented in two succeeding papers (Johnson and Reid; Johnson and Holdsworth, to be published).

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IRON STORAGE AND TRANSPORT AFTER ADMINISTRATION OF VIABLE ERYTHROCYTES TO THE ALBINO RAT

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(*Accepted for publication 4th May, 1961.*)

SUMMARY.

Viable rat erythrocytes were administered intraperitoneally in two dosage levels to male albino rats. The changes in blood haemoglobin, plasma iron and total iron binding capacity (T.I.B.C.) and liver and spleen storage iron were followed for 180 days after giving the erythrocytes. Polycythaemia persisted for at least 20 days and was accompanied by elevation of the plasma iron concentration and depression of the T.I.B.C. During the first 20-60 days liver and spleen storage iron values increased markedly, but thereafter gradually declined. The increase in storage iron concentration was greater in the spleen than in the liver, and in the spleen relatively less iron was found in the ferritin fraction than in the haemosiderin fraction when compared with the values for control rats.

INTRODUCTION.

Under normal circumstances most of the iron released from haemoglobin upon destruction of erythrocytes by the cells of the reticulo-endothelial system is carried in the blood plasma to the bone marrow where it is re-utilized for haemoglobin synthesis. When repeated blood transfusions are given iron is released in the reticulo-endothelial cells more rapidly than it can be utilized by the bone marrow and hence it accumulates in the reticulo-endothelial system. The distribution of this excess storage iron has been studied mainly by histochemical techniques and by chemical analysis in only a few instances, in man in cases of transfusional haemosiderosis (Oliver, 1959) and in experimental animals after repeated blood transfusions (Rous and Oliver, 1918; Krumbhaar and Chanutin, 1922; Finch, Hegsted, Kinney, Thomas, Rath, Haskins, Finch and Fluharty, 1950; Platzer, Young and Yuile, 1955; Brown, Dubach, Smith, Reynafarje and Moore, 1957). However, histochemical techniques are at best semiquantitative and they do not account for all the iron released from haemoglobin into the tissues.

[1] This work was undertaken during tenure of a National Health and Medical Research Council Fellowship.

In the study to be described in this paper rats were transfused with blood from donor animals. Some of the transfused rats were sacrificed at different stages during the resulting polycythaemic state and others at different intervals in the course of recovery from this condition. Plasma iron and total iron binding capacity were measured in addition to the size of the iron stores in the liver and spleen and the relative distribution of iron between the two principal non-haem tissue iron fractions, ferritin and haemosiderin.

Material and Methods.

General plan of the experiment.

Ninety male albino rats aged 14-18 weeks and weighing 240-320 gm. were used. They were divided into five groups, each consisting of 18 rats. From each group six rats received no treatment, while the other 12 were injected intraperitoneally with viable rat erythrocytes. Six of the latter rats each received a total volume of 16 ml. of packed erythrocytes and the remaining six each received a total volume of 32 ml. The total dose of packed cells was subdivided into four equal sub-doses with 10-day intervals between injections in each case. The rats of the five groups were killed 5, 20, 60, 120 and 180 days respectively after the last injection.

Packed rat erythrocytes.

The blood for injection into the recipient rats was obtained by cardiac puncture of donor rats from the same colony. The blood was centrifuged at 1500 *g.* for 10 minutes, the plasma removed, the haemoglobin concentration of the packed erythrocytes determined and the cells injected intraperitoneally into the recipient rats within a few hours of withdrawal.

Collection of materials and analytical methods.

The rats were anaesthetized with ether, the abdomen and chest opened and blood removed from the heart using a syringe and needle previously moistened with heparin solution. The livers and spleens were removed and weighed, and aliquots cut, weighed and then stored at − 16° C. until analysed. A sample of blood was taken for haemoglobin estimation and the remainder was centrifuged at 2,000 *g.* for 15 minutes. The plasma was removed for immediate estimation of iron concentration and T.I.B.C. At the time of death, the peritoneal cavities were free of blood and appeared normal in all rats except in three animals which had received 32 ml. packed erythrocytes and were killed five days after the last injection. In these rats a small amount of blood was still present in the peritoneal cavity.

The following analytical methods were used: blood haemoglobin cencentration—Walsh, Arnold, Lancaster, Coote and Cotter (1953); plasma iron concentration and T.I.B.C.—Morgan and Carter (1960); liver and spleen total storage iron—Kaldor, 1954; liver and spleen ferritin iron—Kaldor, 1958. The difference between the total storage iron and ferritin iron in liver and spleen is considered to represent the insoluble or haemosiderin fraction of the storage iron (Kaldor, 1958).

RESULTS.

In Table 1 are shown the ages and mean body weights and liver and spleen weights of the different groups of rats. Injection of packed erythrocytes in the two dosage levels used did not produce significant changes in any of these values.

In Fig. 1 are shown the blood haemoglobin values and in Fig. 2 the plasma iron and T.I.B.C. values. Five days after the last injection of erythrocytes the

mean blood haemoglobin concentrations were markedly elevated and 15 days later they had fallen considerably but were still significantly higher than in the untreated rats ($P < 0{\cdot}01$). However, by the 60th day after the last

TABLE 1.

Mean body weights and liver and spleen weights for control rats and for rats given intraperitoneal injections of packed erythrocytes.

Total dose of erythrocytes (ml.)	Days after last injection	Age (months)	Body weight (gm.)	Liver weight (gm.)	Spleen weight (gm.)
Nil	5	4	297	9·899	0·706
16			277	9·107	0·804
32			287	9·164	0·748
Nil	20	4·5	293	10·319	0·654
16			274	9·437	0·745
32			292	10·558	0·862
Nil	60	6	346	13·216	0·723
16			343	14·760	0·800
32			347	14·725	0·827
Nil	120	8	330	13·398	0·789
16			330	13·577	0·725
32			348	15·425	0·792
Nil	180	10	367	14·855	0·679
16			374	15·601	0·882
32			358	15·067	0·837

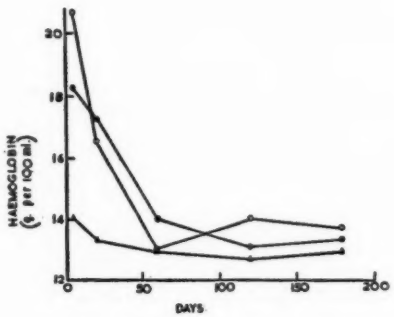

Fig. 1. Changes in blood haemoglobin concentration in rats following the intraperitoneal administration of viable erythrocytes. The last injection of erythrocytes was given on day 0. Each point represents the mean value for six rats.

▲ controls
● 16 ml. erythrocytes
○ 32 ml. erythrocytes

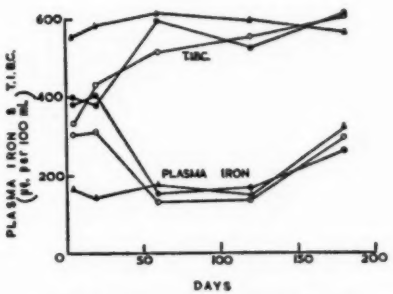

Fig. 2. Changes in plasma iron concentration and T.I.B.C. in rats following the intraperitoneal administration of viable erythrocytes. The last injection of erythrocytes was given on day 0. Each point represents the mean value for six rats.

▲ controls
● 16 ml. erythrocytes
○ 32 ml. erythrocytes

erythrocyte injection and thereafter no significant difference was found between results for control and treated animals. In the rats with elevated haemoglobin values the mean plasma iron concentration was also significantly increased ($P < 0{\cdot}01$), but the T.I.B.C. was significantly diminished ($P < 0{\cdot}01$) with respect to control values. The plasma iron and T.I.B.C. values returned to the control level at the same time as did the haemoglobin concentration.

On the 5th day after the last injection the blood haemoglobin and plasma iron values were significantly higher ($P < 0{\cdot}05$, $P < 0{\cdot}05$ respectively) and the T.I.B.C. values significantly lower ($P < 0{\cdot}05$) in the rats given 32 ml. erythrocytes than in those given 16 ml. erythrocytes. On the 20th day significant differences were found only for plasma iron concentration ($P < 0{\cdot}01$), which was higher in rats given the smaller dose. On subsequent days no significant differences were found for blood haemoglobin, plasma iron and T.I.B.C. values either between the rats given the two dosage levels or between treated and control rats.

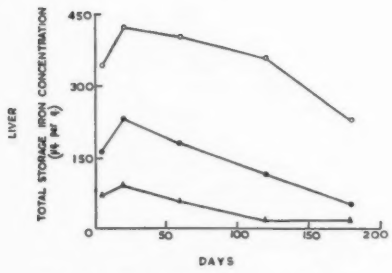

Fig. 3. Changes in liver total storage iron concentration in rats following the intraperitoneal administration of viable erythrocytes. The last injection of erythrocytes was given on day 0. Each point represents the mean value for six rats.

▲ controls
● 16 ml. erythrocytes
○ 32 ml. erythrocytes

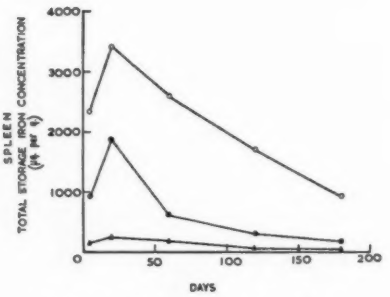

Fig. 4. Changes in spleen total storage iron concentration in rats following the intraperitoneal administration of viable erythrocytes. The last injection of erythrocytes was given on day 0. Each point represents the mean value for six rats.

▲ controls
● 16 ml. erythrocytes
○ 32 ml. erythrocytes

The storage iron values of livers and spleens are presented in Figs. 3 and 4 and Table 2. In both organs the concentrations of storage iron (Figs. 3 and 4) and also the total amounts of storage iron (Table 2—total storage iron content) decreased with age. This was not accompanied by any alteration in the relative distribution of the iron between ferritin and haemosiderin (Table 2—percentage of storage iron in ferritin fraction). In the polycythaemic rats storage iron was increased in both liver and spleen, the increase being approximately twice as great in the rats given the larger dose of erythrocytes. In the

TABLE 2.

Mean liver and spleen storage iron values for control rats and for rats given intraperitoneal injections of packed erythrocytes.

Total dose of erythrocytes (ml.)	Days after last injection	Liver			Spleen		
		Total storage iron concentration (μg. per gm.)	Total storage iron content (mg.)	Percentage of total storage iron in ferritin fraction	Total storage iron concentration (μg. per gm.)	Total storage iron content (mg.)	Percentage of total storage iron in ferritin fraction
Nil	5	73	0·73	83·0	158	0·11	77·3
16		165	1·49	92·6	945	0·76	78·5
32		340	3·14	91·5	2341	1·76	80·9
Nil	20	94	0·99	87·3	251	0·21	85·0
16		233	2·19	93·1	1883	1·43	76·4
32		425	4·51	86·6	3435	3·01	66·1
Nil	60	56	0·73	85·3	185	0·13	78·3
16		180	2·63	93·5	604	0·49	77·0
32		406	5·98	93·1	2602	2·15	72·3
Nil	120	27	0·37	86·5	84	0·06	80·6
16		111	1·52	92·4	327	0·23	62·5
32		362	5·51	93·0	1717	1·31	60·5
Nil	180	26	0·39	79·0	105	0·07	77·5
16		55	0·83	85·0	186	0·16	66·5
32		230	3·43	87·8	973	1·11	50·5

liver maximal total storage iron values were found 60 days after the last injection of erythrocytes while in the spleen maximal values were found 20 days after the last injection. The storage iron values in both organs declined steadily thereafter. The changes in liver storage iron were not accompanied by significant alteration in the relative distribution of the iron between the ferritin and haemosiderin fractions. In the spleen, however, a significant ($P < 0{\cdot}05$) reduction of the proportion of the storage iron in the ferritin fraction was found in the rats on both dosage levels on the 20th day after the last injection of erythrocytes and thereafter. The reduction of the proportion of iron present as ferritin was greater in the rats which had received the larger dose of erythrocytes and in which, as a consequence, total storage iron values were higher.

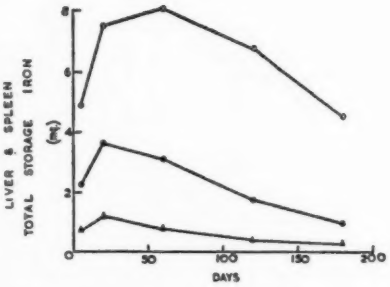

Fig. 5. Changes in the total amount of storage iron found in liver plus spleen in rats following the intraperitoneal administration of viable erythrocytes. The last injection of erythrocytes was given on day 0. Each point represents the mean value for six rats.
▲ controls
● 16 ml. erythrocytes
○ 32 ml. erythrocytes

In Fig. 5 are shown the changes in the sum total of the amount of total storage iron found in the liver plus that found in the spleen. In the animals given 16 ml. erythrocytes maximal values were obtained 20 days after the last injection while in those given 32 ml. maximal values were found on the 60th day.

DISCUSSION.

Following intraperitoneal injection of viable erythrocytes to the rat the red cells rapidly enter the blood stream mainly *via* the right lymphatic duct without alteration in their appearance and with little evidence of haemolysis or phagocytosis (Courtice, Harding and Steinbeck, 1953). It has been shown that in the rabbit intraperitoneally injected erythrocytes have a normal life span after entering the circulating blood (Hollingsworth, 1954). In the present work an increase in blood haemoglobin concentration persisted for at least 20 days after the last injection of erythrocytes, indicating that a large proportion of the cells had remained viable. Since the normal life span of rat erythrocytes is 50-60 days (Berlin, Waldmann and Weissman, 1959) return of the haemoglobin level to that of the controls by the 60th day after giving the blood was as expected. On the 5th day after the last injection the blood haemoglobin concentration was significantly higher in the rats given 32 ml. than in those given 16 ml. erythrocytes, but the concentration fell more rapidly in those given the larger dose. The reason for this may have been that absorption from the peritoneum was slower in the rats given the larger dose (since blood was still found there

in some of the 5-day rats) and that prolongation of the time during which the cells were retained in the peritoneum was associated with greater damage to the erythrocytes and consequent reduction in survival time.

During the period of polycythaemia a marked increase in plasma iron and decrease in T.I.B.C. values was observed. In man a depression of T.I.B.C. occurs in transfusional haemosiderosis and also in other conditions associated with increased iron stores, such as haemolytic and aplastic anaemia and haemochromatosis (Laurell, 1952). In the present experiment the iron stores were increased to the same degree after return of the blood haemoglobin and T.I.B.C. values to the control levels as they were when the haemoglobin and T.I.B.C. values were elevated and T.I.B.C. depressed. This suggests that in the rat the size of iron stores does not have an important influence on the plasma T.I.B.C., but that there may be an inverse relationship between T.I.B.C. and haemoglobin levels. A similar conclusion to this was reached as a result of experiments on rats with iron deficiency anaemia or combined iron and copper deficiency (Morgan, 1961). Plasma iron concentration was increased only during the period of polycythaemia. Hence it is more probable that plasma iron was increased due to increased erythrocyte destruction and/or decreased erythropoiesis than to the increase in the amount of liver and spleen storage iron.

In the rats killed on the 5th and 20th days after the last injection of either dose of erythrocytes the amounts of storage iron in liver and spleen in excess of the amounts found in the corresponding control animals were approximately equal (Table 2). By the 60th day, however, the amounts of storage iron in excess of control values had increased in the liver but decreased in the spleen. This suggests that, initially, storage iron deposition and probably erythrocyte destruction occurred approximately equally in liver and spleen but later a redistribution of the storage iron from spleen to liver occurred. A similar redistribution of storage iron from spleen to liver has been demonstrated in the rabbit after iron loading with saccharated iron oxide (Shoden and Sturgeon, 1958).

As the spleen total storage iron concentration rose to values in the vicinity of 2,000 μg. per gm. the relative proportion of iron found in the ferritin fraction decreased (Table 2). However, in the liver where storage iron concentration did not reach such high values, the distribution of iron remained unchanged. Shoden, Gabrio and Finch (1953) in man and the rabbit, Shoden and Sturgeon (1958, 1959) in the rabbit, and Kaldor (1958) in the rat and mouse found similar changes in storage iron distribution when the total concentration rose to values in excess of 2,000 μg. per gm. Of notable interest in the present work was the finding that in the spleen, the relative amount of storage iron in the ferritin fraction remained low for up to 180 days after giving the blood despite the fact that the total storage iron values steadily declined (Table 2). This suggests that iron was released proportionately from both ferritin and haemosiderin and that transformations of one compound into the other had not occurred.

The total amounts of iron given as haemoglobin to each of the rats on the two dosage levels were 13 and 26 mg. Only approximately one-third of this was recovered in the liver and spleen. Some iron may have been lost due to intravascular haemolysis and renal excretion of the haemoglobin. However, the experimental evidence of Courtice *et al.* (1953) and Hollingsworth (1954), the maintenance of elevated haemoglobin levels for a considerable time after giving the blood and the absence of any gross haematuria suggest that this is unlikely to account for much of the iron lost. Some iron was undoubtedly deposited in tissues other than liver and spleen, probably in the bone marrow and kidneys, but this is unlikely to account for more than a small part of the iron deficit. The third mechanism by which iron could have been lost is that of a disturbance of the balance between iron absorption and iron excretion. The two main factors involved in the regulation of iron absorption are the rate of erythropoiesis and the level of iron stores (Saylor and Finch, 1953; Bothwell, Pirzio-Biroli and Finch, 1958; Seki, Field and Chalmers, 1959; Krantz, Goldwasser and Jacobson, 1959). The polycythaemia, present for at least 50 days in the present experiment, would produce inhibition of erythropoiesis (Elminger, Huff and Oda, 1952) and hence a decrease in iron absorption as would the increase in iron stores. Hence the daily gain of iron from the diet would have been reduced in treated rats throughout the period of the experiment but to the greatest degree in the first 50 days or more when erythropoiesis was diminished. Furthermore, Stevens, White, Hegsted and Finch (1953) and Chapelle, Gabrio, Stevens and Finch (1955) have shown that the daily loss of iron from the body of the mouse was proportional to the total amount of iron present in the body. If this also applies to the rat, then increased iron excretion would have occurred in the treated rats. It is likely, then, that during the experimental period iron excretion was in excess of absorption and that this played some part in causing the difference between injected and recovered iron and also in the decrease in storage iron with the passage of time.

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IRON STORAGE AND TRANSPORT IN IRON-DEPLETED RATS, WITH NOTES ON COMBINED IRON AND COPPER DEFICIENCY

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SUMMARY.

Adult albino rats of both sexes were subjected to repeated bleeding from the tail over a period of eight weeks while being fed a standard diet or a low iron milk diet. This produced anaemia, reduction of plasma iron concentration, elevation of plasma total iron binding capacity and reduction of storage (non-haem) iron present in liver and spleen. The sex difference for plasma iron and storage iron values was maintained in the presence of severe iron deficiency. Complete removal of all storage iron from liver and spleen was not achieved but no definite lower limit for storage iron concentrations in these organs could be demonstrated. The reduction in storage iron values was accompanied by little change in the relative distribution of the iron between ferritin and haemosiderin.

INTRODUCTION.

In man and in laboratory and domestic animals a chronic deficiency of dietary iron intake or chronic loss of iron from the body through haemorrhage leads to progressive depletion of iron from the liver and spleen. These organs normally hold most of the body's accumulated reserves of iron in the form of two non-haem protein compounds, ferritin and haemosiderin, and from these iron is readily released when required elsewhere for synthesis of the essential haem proteins. However, even in the most severe cases of iron deficiency this non-haem "storage iron" does not completely disappear from the liver and spleen (Bogniard and Whipple, 1932; Hahn and Whipple, 1936; Wakeham and Halenz, 1936; Scott and McCoy, 1944; Hallgren, 1954). The remaining non-haem iron has been called "parenchyma iron" (Bogniard and Whipple, 1932; Hahn and Whipple, 1936) and it has been claimed that "parenchyma iron" represents an iron fraction which is physiologically distinct from "storage iron" and furthermore that its presence is essential for the normal functioning of the tissues.

[1] This work was undertaken during tenure of a National Health and Medical Research Council Fellowship.

The main purpose of the experiments to be described in this paper was to study the disappearance of non-haem iron from the liver and spleen of rats in which iron deficiency of varying severity had been induced by haemorrhage alone or in combination with a low-iron diet. Total non-haem (storage) iron and the distribution of iron between ferritin and haemosiderin fractions were measured in the tissues. In addition, blood haemoglobin, plasma iron and plasma total iron binding capacity (T.I.B.C.) were also measured. Because of the existence of distinct sex differences in iron metabolism in the rat both male and female animals were used in the experiments.

Materials and Methods.

Adult albino rats of both sexes, 16 to 20 weeks old were used. The male rats weighed 270-350 gm. and the female rats weighed 140-200 gm. They were fed either the standard laboratory diet of rat cubes and tap water *ad libitum* or else a powdered milk-starch diet with added copper (see below) and distilled water, also given *ad libitum*. Chronic haemorrhagic anaemia was produced by repeated bleeding from the tail.

Iron depletion. Six groups of male rats and four groups of female rats were used. Each group consisted of six rats. The groups will be designated as shown in Table 1. One group of males (M1) and one of females (F1) were fed the standard laboratory diet while a second group of males (M2) and a second group of females (F2) were fed the milk diet. Animals of these four groups were not bled and were used to obtain control values. Rats of the remaining groups were bled from the tail at intervals of 2-4 days for a total period of 8 weeks. The interval between bleedings was varied depending on the desired degree of severity of the iron deficiency. The amount of haemoglobin removed at each bleeding was estimated and from this the cumulative iron loss was calculated. The blood haemoglobin concentration was checked at 4-7-day intervals. Three groups of male rats (M3, M4, M5) were subjected to chronic losses of blood of differing degree and were fed the standard

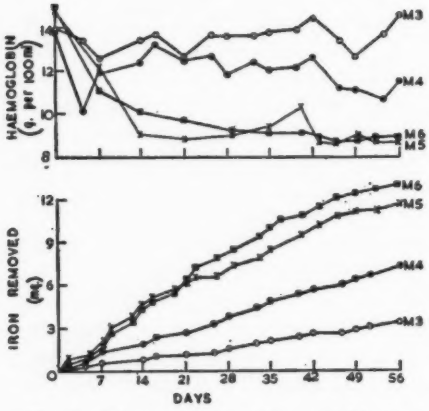

Fig. 1. Changes in blood haemoglobin concentration and cumulative total of iron removed from male rats by repeated bleeding from the tail.

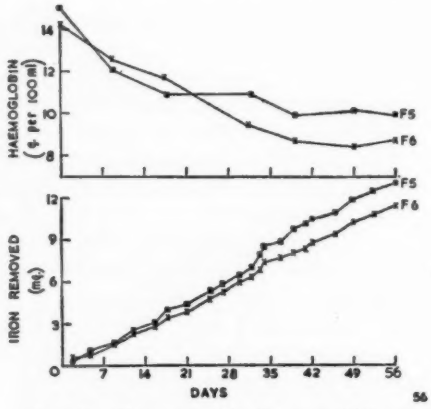

Fig. 2. Changes in blood haemoglobin concentration and cumulative total of iron removed from female rats by repeated bleeding from the tail.

diet, while the last group of male rats (M6) was fed the milk diet and bled to a degree sufficient to keep the blood haemoglobin concentration at a level similar to that of the rats which were kept on the standard diet and subjected to the most severe degree of bleeding (M5). One group of female rats kept on the standard diet (F5) and another group on the milk diet (F6) were also subjected to repeated bleeding. The changes in mean blood haemoglobin concentration and in the cumulative total of iron removed during the course of the experiment are shown in Figs. 1 and 2.

In a preliminary experiment combined copper and iron deficiency was inadvertently induced through omitting the copper supplements from the milk diet. The results obtained on the combined iron and copper-deficient rats will not be presented in detail, but brief reference to them will be made in the Discussion section of this paper.

Milk diet. The milk diet consisted of 70 p.c. dried whole milk, 29 p.c. purified wheat starch and 1 p.c. calcium carbonate (A.R.). Copper was added to this mixture in the proportion of 80 mg. cupric sulphate per kg. milk diet.

Haemorrhage from the tail. Under light ether anaesthesia the ventral tail vein was pieced with a spring-loaded needle and the tail plunged into a tube graduated at 50 ml. and containing warm 0·04 p.c. ammonia solution. Free bleeding for a few minutes usually resulted and could be stopped when required by pressure over the bleeding point. The volume of diluted blood in the tube was made up to the 50 ml. mark, the solution suitably further diluted and the haemoglobin concentration determined absorptiometrically.

Collection of materials and analytical methods. The rats were anaesthetized with ether, the abdomen and chest opened and blood removed from the heart using a syringe and needle previously moistened with heparin solution. The livers and spleens were removed, weighed, and aliquots cut, weighed and stored at $-16°$ C. until analysed. Samples of blood were taken for haemoglobin estimations and the remainder of the blood was centrifuged at 2,000 *g.* for 15 minutes. Plasma iron and T.I.B.C. estimations were performed immediately. The following analytical methods were used: blood haemoglobin concentration—Walsh, Arnold, Lancaster, Coote and Cotter (1953); plasma iron and T.I.B.C.—Morgan and Carter (1960); liver and spleen total storage iron and ferritin iron—Kaldor (1954a, 1958).

RESULTS.

Chronic haemorrhagic anaemia—iron deficiency. In Table 1 are shown the results for body weight, haemoglobin, plasma iron and T.I.B.C. in male and female rats. In the male rats chronic haemorrhage of a degree insufficient to produce a reduction in blood haemoglobin concentration (Group M3) was associated with a significant reduction in plasma iron concentration ($P < 0·01$) but with no significant change in plasma T.I.B.C. ($P > 0·05$). With chronic haemorrhage of a greater severity blood haemoglobin concentration was reduced in rats of both sexes and this was associated with marked reduction in plasma iron concentration and elevation of T.I.B.C. (Groups M4, M5, M6, F5 and F6). The plasma iron values were uniformly higher in females than in males of corresponding groups, but there was no significant sex difference in plasma T.I.B.C.

The storage iron values for liver and spleen are summarized in Table 2. The mean liver weights of the unbled rats fed the milk diet (Groups M2 and F2) were less than the values for corresponding rats fed the standard diet (Groups M1 and F1). However, the mean storage iron concentrations were

TABLE 1.

Mean body weights, blood haemoglobin concentration, plasma iron concentration and total iron binding capacity for control rats and rats subjected to repeated haemorrhage.

Designation	Diet	Total iron removed(mg.)	Initial body weight (gm.)	Final body weight (gm.)	Blood haemoglobin concentration (gm. per 100 ml.)	Plasma iron concentration (μg. per 100 ml.)	Total iron binding capacity (μg. per 100 ml.)
Male rats:							
M1	Standard	0	297·1	331·7	13·92	196·0	507·3
M2	Milk	0	328·8	328·7	13·87	197·3	526·5
M3	Standard	3·3	319·8	350·6	14·60	105·0	534·6
M4	Standard	7·4	307·5	339·6	11·74	64·4	623·8
M5	Standard	11·1	327·7	322·5	8·15	55·3	680·5
M6	Milk	13·0	310·7	319·0	8·60	53·8	725·8
Female rats:							
F1	Standard	0	170·3	191·0	13·37	247·8	566·7
F2	Milk	0	189·0	197·0	12·90	253·5	458·7
F5	Standard	13·1	182·7	209·0	8·40	83·0	712·5
F6	Milk	10·9	182·2	198·8	7·80	95·8	736·5

TABLE 2.

Mean liver and spleen storage iron values for rats subjected to repeated bleeding over a period of eight weeks.

	Liver				Spleen			
Designation	Weight (gm.)	Total storage iron concentration (μg. per gm.)	Total storage iron content (mg.)	Percentage of storage iron in ferritin fraction	Weight (gm.)	Total storage iron concentration (μg. per gm.)	Total storage iron content (mg.)	Percentage of storage iron in ferritin fraction
Male rats:								
M1	12·83	78·5	0·99	86·7	0·77	296·5	0·22	79·8
M2	9·43	120·0	1·14	94·5	0·68	354·2	0·25	85·3
M3	10·46	43·6	0·46	80·6	0·82	101·2	0·08	79·8
M4	9·99	19·0	0·19	75·4	0·91	57·4	0·05	79·6
M5	11·42	18·8	0·21	86·0	1·35	35·2	0·05	85·8
M6	8·68	15·7	0·14	81·3	0·83	42·8	0·03	90·0
Female rats:								
F1	7·38	214·7	1·60	92·5	0·54	683·7	0·36	80·3
F2	6·05	333·8	2·00	91·5	0·44	723·3	0·30	84·3
F5	8·28	75·3	0·63	91·7	1·21	55·3	0·07	79·2
F6	6·40	33·2	0·22	78·5	0·77	52·5	0·04	80·5

higher in the livers of the rats on the milk diet so that no significant difference was found between the total amounts of storage iron present in livers or spleens of unbled rats of the same sex fed either diet. Haemorrhage produced a marked reduction in total storage iron concentrations in liver and spleen in both sexes even where frank anaemia did not develop (Group M3). In both sexes the liver storage values were lower in rats on the milk diet than in rats on the standard diet when both were bled to approximately equal degrees (Groups M5 and M6 and Groups F5 and F6). The relative distribution of iron between ferritin and haemosiderin remained unaltered in the face of variations in total storage iron except in the case of the livers of bled rats on the milk diet (Groups M6 and F6) where the proportion in ferritin was significantly reduced ($P < 0{\cdot}05$). In the unbled rats there was a marked sex difference in storage iron with much higher values for both liver and spleen in the female rats and this difference was maintained even after severe iron depletion.

DISCUSSION.

The results of the experiments reported in this paper failed to demonstrate a lower limit for liver and spleen storage iron values. The rats of both sexes subjected to iron depletion of the greatest severity (bled rats fed low iron diet) showed lower storage iron values than rats subjected to less severe iron depletion. Whether iron depletion of even greater severity would have further lowered storage iron is not known. It does seem unlikely, however, that storage iron could be completely removed from either liver or spleen. The circulating erythrocytes at the end of their life span are destroyed in the cells of the reticuloendothelial system, mainly in the liver and spleen. This destruction of erythrocytes involves catabolism of haemoglobin, with the formation of the non-haem or storage iron compounds, ferritin and haemosiderin. If the erythrocyte life span remains unchanged in haemorrhagic anaemia then the rate of destruction of erythrocytes should be approximately proportional to the blood haemoglobin level and hence the liver and spleen storage iron might be expected to be lower in animals with a lower blood haemoglobin concentration. Such was found to be the case in the present investigation.

A sex difference in iron metabolism has been found in the rat for plasma iron concentration (Kaldor, 1954b) and for storage iron levels (Steenbock, Semb and Van Donk, 1936; Widdowson and McCance, 1948, 1953; Kaldor and Powell, 1957) both of which are higher in the female animals. The present investigation confirmed these differences and showed that they persist in the presence of severe iron depletion due to repeated haemorrhage. The reasons for the sex differences are uncertain, but they may be due to endocrine causes since the difference in iron stores only becomes apparent with attainment of sexual maturity and castration of female rats results in decreased storage iron (Steenbock *et al.*, 1936; Widdowson and McCance, 1948).

TABLE 3.

Mean blood haemoglobin, plasma iron and total iron binding capacity, storage iron and liver copper values for control rats and rats bled while being fed a copper deficient diet.

Treatment	Number of rats	Total iron removed (mg.)	Blood haemoglobin concentration (gm. per 100 ml.)	Plasma iron concentration (μg. per 100 ml.)	Total iron binding capacity (μg. per 100 ml.)	Total storage iron content of liver plus spleen (mg.)	Liver copper concentration (p.p.m. dry weight)
Untreated controls, standard diet	4	0	14·88	145·8	491·3	0·93	16·7
Repeated bleeding, low copper diet	4	2·5	9·45	44·3	715·3	1·15	
Repeated bleeding, low copper diet	4	5·8	5·96	21·6	839·8	0·93	4·1

The depletion of liver and spleen storage iron produced by the repeated removal of blood occurred with little alteration in the relative proportion of the iron in the ferritin and haemosiderin fractions. Mobilization of storage iron proportionately from ferritin and haemosiderin has been observed in rabbits bled after being given colloidal iron intravenously (Shoden, Gabrio and Finch, 1953; Konitzer, 1957). These results and those of the present investigation indicate that iron stored as either ferritin or haemosiderin is equally readily released when required for haemoglobin synthesis.

In the preliminary experiments where male rats were fed a milk diet not supplemented with copper and were bled twice weekly from the tail for a period of eight weeks anaemia developed more quickly and was of greater severity than in rats bled while being given the milk diet with added copper (M6). The results for the rats with combined iron and copper deficiency are summarized in Table 3. It can be seen that the rats which were bled most severely while on the low copper diet had lower haemoglobin and plasma iron values and higher T.I.B.C. values than the most severely bled rats (M6) on the milk diet supplemented with copper. Despite this the amount of storage iron found in the liver and spleen was no less than that found in control rats fed the standard diet and not subjected to bleeding. The lower copper values found in the bled rats are of the same order as values generally found in pure copper deficiency (Underwood, 1956). The low haemoglobin and plasma iron values found in the presence of adequate liver and spleen storage iron suggest that mobilisation of storage iron and/or utilization of iron for haemoglobin synthesis is depressed. Similar conclusions on the effects of copper deficiency were reached by Gubler, Lahey, Chase, Cartwright and Wintrobe (1952) as a result of their experiments with copper-deficient swine.

It is well known that in man plasma iron concentration decreases and T.I.B.C. increases in iron deficiency anaemia (Laurell, 1952) while in swine similar changes are found in either iron or copper deficiency (Cartwright, Gubler, Lahey and Chase, 1952). The present results show that in the rat, also, iron deficiency or combined deficiency of iron and copper are accompanied by low plasma iron and high T.I.B.C. values. In pure iron deficiency the low plasma iron concentration is undoubtedly due to depletion of the iron present in the body, while in combined iron and copper deficiency it is probably due to defective mobilisation of storage iron and inefficient iron absorption from the diet (Gubler *et al.*, 1952). The mechanism producing the increase in T.I.B.C. is less clearly understood. It may be noted that in both pure iron deficiency (Table 1) and in combined iron and copper deficiency (Table 3) an increase in T.I.B.C. occurred only in those rats with reduced haemoglobin values, and that, in general, the greater the reduction in haemoglobin, the greater was the increase in T.I.B.C. The plasma T.I.B.C. may therefore be in some way inversely related to the blood haemoglobin concentration. It would appear unlikely that the size of the iron stores is important in regulating the plasma

T.I.B.C. because (i) a decrease in iron stores without accompanying anaemia was not associated with a change in T.I.B.C. (Tables 1 and 2—Group M3) and (ii) the T.I.B.C. was greatly increased in combined copper and iron deficiency despite the fact that considerable storage iron remained in the liver and spleen (Table 3).

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THE MECHANISM OF ABSORPTION OF SUBCUTANEOUS EXTRAVASATIONS OF BLOOD[1]

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SUMMARY.

The rate and mechanism of absorption of blood from subcutaneous extravasations has been investigated in rats. Absorption of intact red cells from the peritoneal cavity is rapid, but in the subcutaneous tissues red cells are mainly broken down *in situ* with absorption of catabolised products. A difference in the coagulation and lytic mechanisms in the peritoneal and subcutaneous tissues seems to account for the different absorption mechanisms. Simultaneous injections of anticoagulants, hyaluronidase, and streptokinase did not increase the absorption of red cells. A large percentage of the Fe^{59} or Cr^{51} from broken-down red cells passed to the liver.

INTRODUCTION.

Extravasations of blood into tissues are removed at varying rates from different parts of the body. Both red cells and plasma are rapidly absorbed from the peritoneal cavity (Courtice, Harding and Steinbeck, 1953; Hahn, Miller, Robscheit-Robbins, Bale and Whipple, 1944) mainly by the diaphragmatic lymphatics. This is the basis of intraperitoneal transfusions in infants and children and of auto-transfusion in ruptured ectopic gestation. On the other hand, a subcutaneous haematoma may be disfiguringly apparent for ten days or more. The mechanism of removal of blood and plasma following subcutaneous injections has been investigated in rats with the aid of radio-isotopes and it has been found that the majority of the red cells are catabolised *in situ* and are not absorbed intact. The results also suggest that the different rate and mechanism of absorption is due to a different response of the blood coagulation mechanism in the two sites.

METHODS AND MATERIALS.

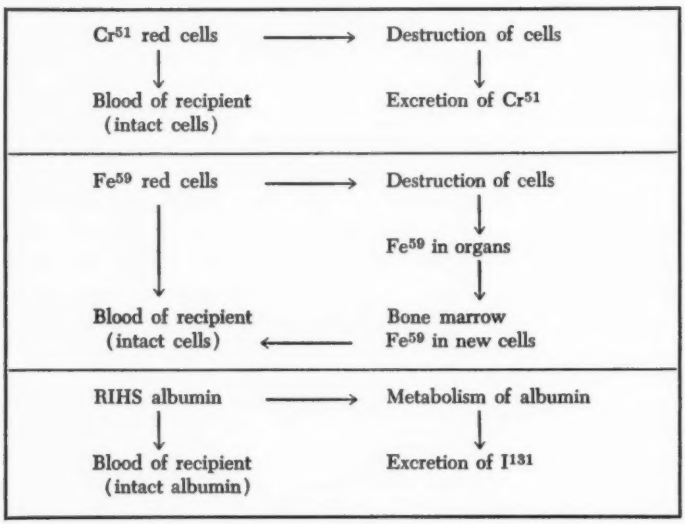
Principle.

All experiments were performed on albino rats. Red cells injected subcutaneously or intraperitoneally were labelled with Cr^{51} or Fe^{59} and, when required, radio-iodinated human serum albumin (RIHSA) was added to plasma. Following the injection of tagged material,

[1] The radio-isotopes used in this work were obtained with the aid of a grant from the National Health and Medical Research Council of Australia.

the amount absorbed from the site of injection was measured at different time intervals by radio-assay of the blood and of the perfused liver, spleen and kidneys of the recipient animal. The total amount of absorbed material in the blood was calculated from the radio-assay value of an aliquot of peripheral blood and the blood volume calculated from the body weight (67 ml. of blood per kg. of body weight).

When red cells were labelled with Cr^{51} prior to injection, it was assumed that any radioactivity in the blood of the recipient would be due to red cells which had been absorbed intact from the injection site. The chromium of red cells catabolised at the site of injection and after absorption is rapidly excreted. The fate of the iron in red cells is rather different. Labelled cells absorbed intact from the injection site are found in the blood of the recipient. Those cells which are catabolised *in situ* yield iron which passes immediately to various organs. Possibly after further breakdown this iron may be utilised by developing erythrocytes and appear in the circulation. Thus circulating Fe^{59} may be in red cells absorbed intact or in newly formed red cells which have incorporated the Fe^{59} from catabolised cells. On the other hand, Fe^{59} in the perfused organs must have been derived from catabolised injected cells. RIHSA in the blood of the recipient could have been derived only from absorbed albumin. The I^{131} of metabolised RIHSA is rapidly excreted. The fate of the various isotope-labelled substances is summarised below:



Isotopes.

Cr^{51} and Fe^{59} of high specific activity were obtained from the Abbott Laboratories, Chicago, through the Commonwealth X-Ray and Radium Laboratory, Melbourne. The chromium was supplied as sodium chromate and the iron as ferric chloride. RIHSA was obtained through the same authority from the Radiochemical Centre, Amersham, England.

Chromium labelling.

Blood was obtained from the exposed hearts of donor rats with a needle and syringe containing a small amount of heparin. The blood was centrifuged, the supernatant plasma removed and the Cr^{51} solution in 2 ml. of physiological saline added to the cells (approxi-

mately 1·5 μc. of Cr^{51} per 1 ml. of cells). The cells were placed in an incubator at 37° C. for 60 minutes and shaken every 15 minutes. They were then washed three times with physiological saline solution and the plasma replaced after the last washing. A standard of 0·5 ml. of this blood in 20 ml. of distilled water was used in the radio-assays.

Iron labelling.

Donor rats were each injected intravenously with approximately 10 μc. of Fe^{59} solution. Three to four weeks later, when the circulating red cells contained the maximum amount of Fe^{59}, blood was collected from the hearts and pooled. A standard of the blood was also prepared for the radio-assays. In some experiments the red cells were haemolysed by freezing and thawing three times.

Plasma and serum labelling.

Blood from donor rats was allowed to clot and the serum removed. An equal amount of blood from other rats was mixed with heparin and the plasma obtained. RIHSA (0·5 ml.) was added to 9·5 ml. of both the serum and the plasma.

Injection of material.

Intraperitoneal injection was used as the basis of all comparisons. In the early experiments subcutaneous injections were made into the thigh and over the anterior abdominal wall, but as no difference was found, the results were combined. In later experiments all injections were given into the abdominal subcutaneous tissues.

Radio-assay.

Collection of blood samples and perfusion and preparation of organs for radio-assay were performed as described by Brading, George and Walsh (1956). All radio-assays were made with an EKCO scintillation counter with an "end-on" crystal arrangement.

RESULTS.

Absorption of cells labelled with Cr^{51}.

The appearance of labelled red cells in the blood following intraperitoneal and subcutaneous injection of 1·0 ml. of blood is shown in Fig. 1. Each value

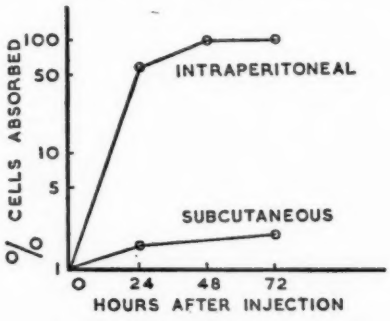


Fig. 1. Absorption of Cr^{51}-labelled red cells into the blood stream from the peritoneal cavity and from the subcutaneous tissues.

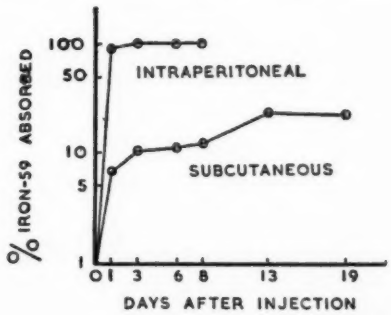


Fig. 2. Absorption of Fe^{59} from labelled red cells following intraperitoneal and subcutaneous injections.

is the mean of results from four rats. It can be seen that there is rapid absorption of cells injected intraperitoneally and these cells remain intact in the circulation. By contrast a maximum of 1·9 p.c. of the subcutaneously injected Cr^{51} was found in the blood and organs of the animals at 72 hours. Although it is not shown in the Figure, it is also significant that the bulk of the recovered Cr^{51} was found in the kidneys and livers of these animals. For example, at seven days 33 p.c. of the Cr^{51} was found in the kidneys (presumably the result of glomerular excretion of labelled haemoglobin) and 40 p.c. in the liver, the significance of which will be discussed later.

Absorption of cells labelled with Fe^{59}.

The rapid absorption of red cells injected into the peritoneal cavity was confirmed by the studies with Fe^{59}-labelled red cells. It can be seen from Fig. 2 that almost 100 p.c. of the injected cells were found in the blood stream within 24 hours, but that the Fe^{59} in the cells injected subcutaneously was more slowly absorbed. Approximately 11 p.c. was found in the blood and organs on the sixth day and 23 p.c. by the thirteenth day. These figures are greater than corresponding figures for the Cr^{51}-labelled cells, but this is due to the difference in metabolism of Cr^{51} and Fe^{59}.

TABLE 1.

Absorption of iron-59 from blood injected intraperitoneally or subcutaneously. (Mean value).

Route of injection	Time after injection (days)	Number of animals	Percentage distribution of absorbed iron			
			Blood	Liver	Kidneys	Spleen
Intraperitoneal	1	4	98·1	0·8	0·4	0·8
	3	5	98·1	1·0	0·2	0·7
	6	2	95·5	2·6	0·5	1·5
	8	3	96·6	1·6	0·1	1·8
Subcutaneous	1	5	23·0	66·0	4·6	6·4
	3	6	45·1	51·9	0·9	2·1
	6	6	49·9	46·3	1·4	2·4
	8	6	50·2	47·9	0·4	1·5
	13	5	45·4	51·5	1·4	1·6
	19	6	55·1	42·1	1·2	1·6

The relative distribution of the Fe^{59} in the blood and tissues is shown in Table 1. The amount found in the liver following subcutaneous injection was high and contrasts with 6-10 p.c. deposited in the liver following oral administration or intravenous injection of Fe^{59}, as found in earlier work (Brading *et al.*, 1956). A smaller percentage was found in the kidneys than when the cells

were labelled with Cr^{51} but the absolute amounts were almost identical. On the other hand, more Fe^{59} was found in the blood at comparable time intervals after injection. This was the result of re-utilisation of iron from catabolised haemoglobin. The difference between the total amounts of Fe^{59} and of Cr^{51} recovered in the blood and organs is largely due to the re-utilisation of iron, the corresponding amount of chromium being excreted in the urine.

Absorption of haemolysed blood.

The results of the previous experiment suggested that red cells injected subcutaneously were broken down at the site of injection and that partially catabolised products, haemoglobin and possibly subcellular fractions, were absorbed. For this reason, haemolysed blood prepared from blood labelled with Fe^{59} was injected subcutaneously and intraperitoneally and the rate of absorption of the Fe^{59} compared with that from the original whole blood. The results are shown in Table 2. Following intraperitoneal injection less than half of the Fe^{59}

TABLE 2.

Absorption of whole and haemolysed blood labelled with iron-59, following intraperitoneal and subcutaneous injections.

Material and route of injection	Percentage absorbed	Time after injection	
		3 days	7 days
Whole blood intraperitoneal	Total	97·9	100
	Found in blood	95·3	95·9
	liver	1·2	2·1
	kidneys	0·3	0·6
Haemolysed blood intraperitoneal	Total	45·6	41·9
	Found in blood	14·4	19·4
	liver	12·2	10·1
	kidneys	17·8	11·3
Whole blood subcutaneous	Total	14·3	11·5
	Found in blood	8·1	7·1
	liver	5·4	3·7
	kidneys	0·2	0·2
Haemolysed blood subcutaneous	Total	27·1	40·4
	Found in blood	6·9	20·4
	liver	17·5	14·0
	kidneys	1·1	5·0

was recovered and there was little difference between the amounts found at three and at seven days. This suggests that the rate of absorption was probably rapid but that more than half was lost, probably by excretion of haemoglobin

through the kidneys. The bulk of the retained portion was found in the blood as re-utilised iron but large amounts were found in the kidneys and liver. The amount of Fe^{59} recovered in the blood and organs after subcutaneous injection was less at three days but almost the same at seven days as after intraperitoneal injection. At three days a greater proportion (64·6 p.c.) was found in the liver than at seven days (34·6 p.c.) or at three days following intraperitoneal injection (26·7 p.c.).

Absorption of RIHSA in plasma and serum.

RIHSA mixed with plasma or serum from donor rats was injected either intraperitoneally or subcutaneously in 0·5 ml. amounts into recipient rats. Each animal received approximately 0·1 mg. of human albumin. The percentage of the injected RIHSA found in the blood of the recipient animals under the different experimental conditions is shown in Table 3. Courtice and Steinbeck

TABLE 3.

Absorption of RIHSA in plasma and serum after subcutaneous and intraperitoneal injections.

Material injected	Route of injection	Time after injection	Number of rats	Percentage of dose in blood of recipient (mean)
Plasma	Subcutaneous	24 hours	4	14·7
		48 hours	4	9·1
		72 hours	4	5·9
Plasma	Intraperitoneal	24 hours	4	20·6
		48 hours	4	10·7
Serum	Subcutaneous	24 hours	4	16·4
		48 hours	4	9·5
		72 hours	4	6·3
Serum	Intraperitoneal	24 hours	4	21·9
		48 hours	4	12·5

(1951) demonstrated that the rat can absorb large amounts of homologous plasma (20 ml./kg. in 3 to 5 hours) from the peritoneal cavity. Although the absorption of fresh heterologous plasma is delayed, absorption of heterologous albumin is not delayed (Courtice *et al.*, 1951; Lake, Simmonds and Steinbeck, 1953). The amounts in the blood following injection diminished rapidly as is seen in Table 3 and the rate can probably be related to the heterologous nature of the labelled protein. However, the significant points from the experiments shown in Table 3 are: (a) that there is no difference in the results between plasma and serum with either route of injection, and (b) that more is found in the blood at the same time intervals after intraperitoneal injection than after

subcutaneous injection. It is not possible to determine from these results the relative rates of absorption from the two injection sites, but in view of the fact that the rate of disappearance from the blood is similar in the two instances, it would seem that there is no impairment of absorption from the subcutaneous tissues.

A further experiment was performed in which RIHSA was added to heparinised whole blood and 1 ml. amounts injected intraperitoneally into four rats and subcutaneously into a further four rats. No significant difference was found in the amount of RIHSA in the blood 24 hours after these injections.

Absorption of different amounts of blood from subcutaneous injections.

For this experiment red cells were labelled with Cr^{51} as before and amounts of 1 ml., 2 ml. and 3 ml. injected subcutaneously and intraperitoneally into groups of rats as shown in Table 4. The amount of Cr^{51} in the blood, liver,

TABLE 4.

Absorption of chromium-51 following injection of various amounts of labelled blood.

Route of injection	Time after injection	Percentage of dose in blood and organs		
		Dose of 1 ml.	Dose of 2 ml.	Dose of 3 ml.
Subcutaneous	24 hours	3·3	4·5	3·5
	48 hours	4·0	3·1	5·2
	72 hours	4·4	3·6	3·3
Intraperitoneal	24 hours	92·0	92·0	91·0
	48 hours	92·0	91·0	100·0

spleen and kidneys of the recipient animals was measured at different time intervals and expressed as a percentage of the injected material. It can be seen that the absorption does not vary within the limits of the doses employed.

Influence of coagulation and fibrinolysis.

It was observed in the course of this work that when blood was injected subcutaneously, coagulation occurred within 15 minutes and remains of the resulting clot were often visible 13 days after injection. In many instances small cysts had formed and contained the liquefied breakdown products of the blood. By contrast, clots were not observed in the peritoneal cavity. The possibility was considered that breakdown of the red cells in the subcutaneous tissue, with slow absorption of their products, might be due to the absence of fibrinolysis in the tissues and its presence in the peritoneal cavity. It was therefore decided

to perform a series of experiments designed to investigate this possibility. The results were as follows:

(i) Between 4 ml. and 5 ml. of blood were removed from the heart of each rat by cardiac puncture and immediately injected intraperitoneally into the same animal. After 30 minutes the abdomen was opened under ether anaesthesia and residual blood removed with a pipette. No clots were observed and the addition of thrombin did not produce coagulation of the removed blood. Plasma obtained from blood removed from the peritoneal cavity was mixed with an equal volume of clotted plasma and placed at 37° C. for 15 hours. Lysis of the fibrin did not occur.

(ii) Blood obtained from the heart of a donor rat was placed in two small tubes 2 in. long × ¼ in. internal diameter. After 15 minutes the resulting clots were freed from the sides of the tubes and placed in the peritoneal cavities of two rats through small laparotomy incisions. The abdominal cavities were opened 18 hours later and only small clots, approximately 10-25 p.c. of the size of the original clots, were found. This suggested that the original clots had undergone some degree of lysis.

(iii) Washed red cells suspended in saline solution were injected subcutaneously into rats. Two hours later a firm clot, indistinguishable from that produced by whole blood, was found in every instance. The fibrous matrix had obviously been supplied from the animal's own plasma.

(iv) Blood labelled with Cr^{51} was divided into four parts, each of 13 ml.

A. 1 ml. of saline solution was added.

B. This part was centrifuged, the plasma removed and replaced with a volume of saline 1 ml. more than the volume of plasma removed.

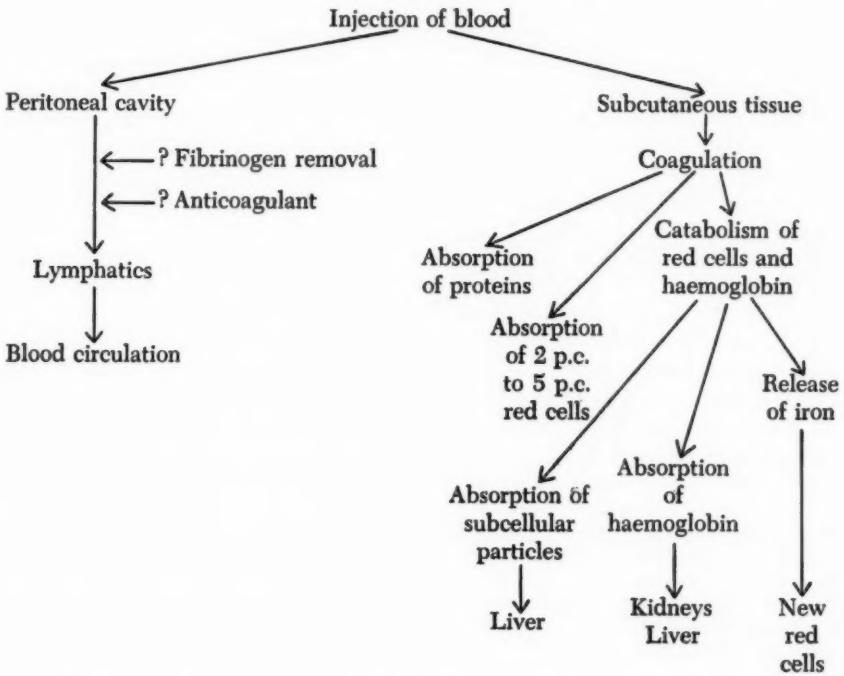
C. 1 ml. of "Hyalase" (B.W. & Co.) containing 3,000 units of hyaluronidase was added.

D. 1 ml. of a solution containing 30,000 units of streptokinase and 12,500 units of streptodornase were added.

Four groups of 12 rats each corresponding to the above preparation were injected subcutaneously with 1 ml. of blood. Four rats from each group were killed 24 hours later and the Cr^{51} in the blood and organs measured. A further four from each group were killed 48 hours after injection and the remainder 72 hours after injection. No differences were found in the amount of Cr^{51} in the blood and organs and the maximum percentage at 72 hours was 3·1. The added enzymes were therefore ineffective in facilitating absorption of intact cells and removal of the autologous plasma was similarly without effect.

DISCUSSION.

A possible interpretation of the findings is shown as a diagram below:



The observed incoagulability of blood removed from the peritoneal cavity could be explained either by a lytic enzyme acting on fibrinogen but not on fibrin, or by the presence of a tissue anticoagulant which is present in excess and which antagonises thrombin, or by precipitation of fibrinogen. The mechanism involved has not been investigated in the present work, but it obviously permits rapid absorption of red cells. Fibrinolysis may have occurred in the peritoneal cavity when clots of blood were placed in the cavity, but if it did so, it was a relatively slow process under the experimental conditions. On the other hand, it was not possible to demonstrate *in vitro* lysis of a clot by adding blood removed from the cavity to clotted plasma.

It is clear from the experiments with red cells labelled with Cr^{51} that few cells enter the circulation intact from the subcutaneous tissues. These observations were confirmed by the experiments with Fe^{59}-labelled cells. On the other hand, there is no evidence from the experiment with RIHSA that there is any impairment of the absorption of proteins. It also seems clear that coagu-

lation of the injected blood, or of plasma from the animal's own circulation which mixed with the injected material, is largely responsible for the failure of absorption of red cells. There was no evidence that the lymphatic vessels themselves were involved in the failure of red cell absorption, but this possibility was not specifically investigated. All the results suggest that there is *in situ* breakdown of injected blood and this is consistent with the well-known colour changes that occur in haematoma.

The deposition of absorbed iron in the liver from blood injected subcutaneously was an unexpected finding in view of the relatively small percentage of orally ingested Fe^{59} found in this organ (Brading *et al.*, 1956). Further, the utilisation of this iron for incorporation into new red cells was slower than was expected. At 19 days after injection, 41 p.c. of the absorbed iron was still in the liver. These findings are consistent with the possibility that absorption of partly degraded subcellular fractions occurs from the site of subcutaneous injection and that catabolism is completed in the reticulo-endothelial system. A certain amount of haemoglobin is certainly absorbed and passes to the kidneys, as is seen in both the Fe^{59}- and the Cr^{51}-labelled cell experiments. Finally, it is possible that some iron is released from the haemoglobin *in situ* and together with that released in the reticulo-endothelial cells is rapidly utilised for the formation of new red cells.

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LOCALIZATION OF 18 S GAMMA-MACROGLOBULIN IN PLASMACYTOID CELLS OF THE BONE MARROW OF A CASE OF MACROGLOBULINAEMIA

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SUMMARY.

18 S macroglobulin was localised by specific fluorescent antibody in plasmablast cells of the bone marrow of a patient suffering from cryogel-macroglobulinaemia. Specific fluorescence was not observed in mature plasmacytes, or in the plasmacytes of the patient's lymph-nodes. The cells showed both cytoplasmic and nuclear fluorescence in contrast to those of a previous case of macroglobulinaemia reported by the author in which only cytoplasmic fluorescence was found in mature and immature bone marrow plasma cells. Other workers have found only nuclear fluorescence in lympho-cytoid cells from a case of macroglobulinaemia. It is suggested that these differences are a reflection of the cytochemical diversity underlying the well-known clinical diversity of macroglobulinaemia.

INTRODUCTION.

Recently (Curtain, 1959), we demonstrated, by the fluorescent antibody technique of Coons and Kaplan (1950) the presence of γ-macroglobulins in the cytoplasm of plasmacytoid cells of the bone marrow of two patients with macroglobulinaemia. Subsequently, Dutcher and Fahey (1960), using the same technique, were able to localize 18 S macroglobulin in the nucleus as well as the cytoplasm of the bone marrow plasma cells in a case of macroglobulinaemia.

This paper describes the localization of a cryogel-macroglobulin in the nucleus and cytoplasm of bone marrow lymphoid cells from a patient with cryogel-macroglobulinaemia.

MATERIALS AND METHODS.

Cryogel-macroglobulin.

This was obtained from a patient presenting with Raynaud's phenomenon. The patient's serum set to a gel at 21° C. and was found to contain 4·3 gm. per 100 ml. of a macroglobulin with a S_{20} of 18 S. Full clinical and other details will be published (Barnett, Sawers and O'Dea). The macroglobulin was isolated by cooling the serum to 5° C., centrifuging and dissolving the precipitate in 0·14 M sodium chloride at 37° C. It was further purified by two cycles of cooling and redissolving in 0·14 M sodium chloride at 37° C.

Bone marrow and lymph node.

Samples of bone marrow were obtained by sternal puncture. Smears were made from the fresh marrow on slides which had been coated with bovine serum albumin (Armour) by dipping in a 1 p.c. solution and drying at 100° C. The slides were stored in iso-pentane at − 70° C.

Lymph node tissue was obtained by biopsy from the cervical and axillary lymph nodes. The pieces of tissue were washed briefly in 0·14 M sodium chloride at 37° C. and then stored in iso-pentane at − 70° C. Frozen sections (5-7 μ) were cut at a knife temperature of − 15° C. in a cryostat similar to that described by Coons, Leduc and Kaplan (1951). The sections were mounted on slides coated with 1 p.c. albumin.

Fluorescent antimacroglobulin.

Specific antibody to the macroglobulin was prepared and purified as described previously (Curtain, 1959) and conjugated to fluorescein-isocyanate by the method of Coons and Kaplan (1950). Low molecular by-products of conjugation were removed by passage of the crude conjugate through a column of "Sephadex" G-25[1] cross-linked dextran gel. Excessively conjugated antibody which would cause non-specific staining was removed by chromatography on di-ethylamino-ethyl-cellulose (Curtain, 1961).

Fluorescence microscopy and treatment of smears and sections.

These in general followed the methods of Coons and Kaplan (1950). Any significant departures in technique have already been described (Curtain, 1959, 1961).

RESULTS.

Lymph-node.

Sections of lymph-node stained with haematoxylin-eosin showed some sinus hyperplasia, but were otherwise normal. After treatment with fluorescent anti-macroglobulin a diffuse specific fluorescence was observed only on the walls of the cortical blood vessels. This was presumably due to precipitation of macroglobulin on the blood vessel walls.

Bone marrow.

The only abnormality in bone marrow smears stained with haematoxylin-eosin was the presence of a fairly large number (1 per 2 high power fields) of cells with a dense nucleus and a thin rim of basophilic cytoplasm. These cells were considered to resemble plasmablasts. There was no increase in the number of mature plasma cells. After treatment with fluorescein-anti-macroglobulin cells which appeared to be the same as the plasmablasts, were observed with a brightly fluorescing uneven rim of cytoplasm and a very unevenly fluorescing nucleus (Fig. 1). After the fields containing fluorescing cells were marked and photographed, the slides were washed in physiological saline for three minutes and exposed to formalin vapour for 30 minutes. They were then stained with haematoxylin-eosin, and re-examined to confirm that the fluorescing cells

[1] Obtained from Pharmacia, Upsala, Sweden.

Fig. 1. Fluorescence photomicrograph of a plasmacytoid cell containing macroglobulin in a bone marrow smear from a case of cryogel macroglobulinaemia. Smear treated with fluorescein-anti-macroglobulin illuminated with light at 366 mμ and photographed through a Wratten No. 15 filter. Magnification of print 1,100.

were in fact the plasmablasts observed earlier (Fig. 2). No fluorescence was observed in control smears of the patient's marrow which had been incubated for 30 minutes at 37° C. and washed briefly in saline before treatment with the fluorescent antibody. This indicated that the fluorescence was specific (Coons and Kaplan, 1950).

In none of the smears was specific fluorescence observed in mature plasma cells. The faint background fluorescence observed in Fig. 1 is due in part to the bluish-grey auto fluorescence of neutrophils and in part to specific staining of macroglobulin apparently adsorbed to the rouleaux of red cells.

DISCUSSION.

The implications of the immuno-cytochemical localizations of abnormal serum proteins have been extensively discussed (Wollensak and Seybold, 1956; Vasquez, 1958; Curtain, 1959; Dutcher and Fahey, 1960). The central problem

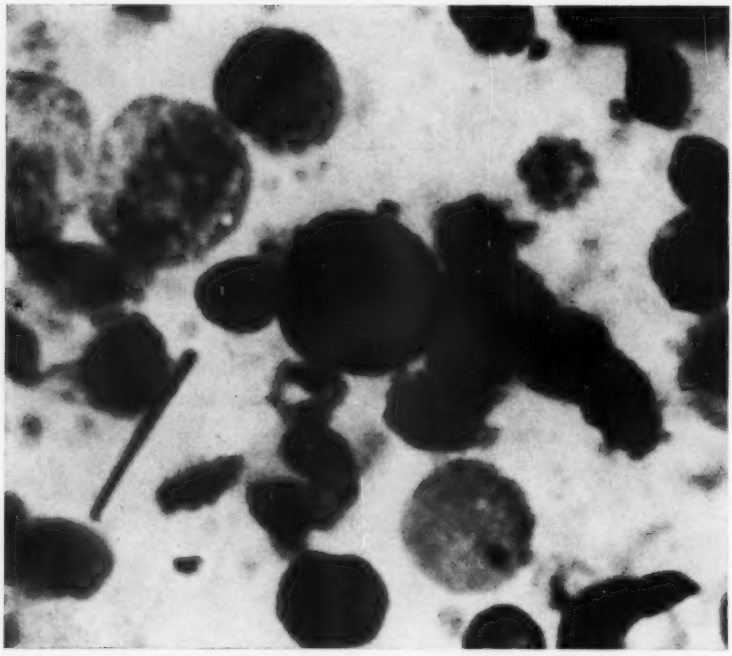

Fig. 2. The smear of Fig. 1 after staining with haematoxylin-eosin. Print magnification 1,100.

is whether the localization of a protein in a particular cell is *a priori* proof that the protein is being synthesised by that cell, or merely being stored there. Macroglobulin has, in fact, been observed in immature and mature plasmacytes (Curtain, 1959). This may be some indication of synthesis during maturation and differentiation of these cells, analogous to the production of antibody in lymph-node plasma cells observed by Coons, Leduc and Connolly (1955). In many dysproteinaemias, however, the cells appear to be at one stage of maturation only. In myeloma these are mature plasma cells and in the classical Waldenström (1958) macroglobulinaemia small lymphocytes with very little cytoplasm.

In the present case macroglobulin-containing cells were found only in the patient's bone marrow, not in his lymph-nodes. The cells also were dissimilar to those of the classical Waldenström syndrome in that they appeared to be plasmablasts. The cells observed by Dutcher and Fahey (1960) also appeared to be plasmacytoid. It is noteworthy that in our case, macroglobulin was not observed in mature plasma cells. If the macroglobulin-containing cells are plasmablasts it is interesting to speculate on their ultimate fate. Do they pro-

duce macroglobulin as plasmablasts, only to cease producing it, in favour of normal 7 S globulins as they mature? This seems contrary to the views currently held on the origins of the cell lines producing abnormal serum proteins (Mackay, 1956; Curtain, 1959). Also against this hypothesis is the absence of a greatly increased number of mature plasma cells, paralleling the increase in plasmablasts. The problem may be better understood if more were known about the synthesis of 18 S macroglobulin which occurs in normal serum in small amounts.

It is difficult to assess the significance of the intra-nuclear fluorescence observed in our case and by Dutcher and Fahey (1960). That observed by the latter was compact, surrounded by a well-defined dark zone and with a diffuse cytoplasmic fluorescence. The nuclear fluorescence in our case is diffuse and uneven. This again is in contrast to our previous finding (Curtain, 1959) of only cytoplasmic fluorescence in a case of macroglobulinaemia with an atypical clinical history. In view of the other variable features observed in macroglobulinaemia it is not unreasonable to suppose that the cytochemical basis of the disease may also be variable. This supposition can only be tested by investigation of many more cases of this rare disease.

Acknowledgments. The author is grateful to Dr. A. J. Barnett and Dr. J. McLean for bringing the case of cryogel-macroglobulinaemia to his notice, for supplying serum and biopsy material, and, with Dr. R. Sawers, clinical pathologist to the Alfred Hospital, for giving opportunities of stimulating discussions. Technical assistance was provided by Miss Joan Harris.

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